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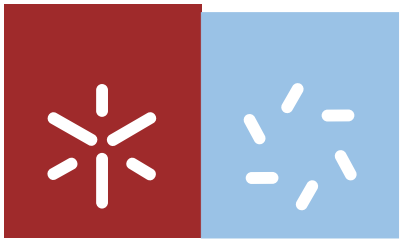

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Development of DODAC/B:MO:DC-Chol lipoplexes as a novel non-viral method for transfection

Dissertação de Mestrado
Mestrado em Genética Molecular

Trabalho realizado sob a orientação da
Professora Doutora Andreia Ferreira de Castro Gomes
E co-orientação da
Professora Doutora Maria Elisabete Cunha Dias Real Oliveira

Outubro de 2012

Acknowledgments

First of all, I would like to thank my supervisors, Dr. Andreia Gomes and Dr. Maria Elisabete Oliveira for their guidance, outstanding patience, remarkable support, kind advice and, most of all, for the trust they bestowed in me and my work throughout my Master research studies.

I would also like to thank João Neves, Ana Oliveira and Isabel Oliveira for all the knowledge they shared and for all the support and good advices in the laboratory.

Additionally, I would like to thank my colleagues who worked with me in the laboratory. Thank you for all the support and for making it an unforgettable experience.

I would also like to thank my parents and my little brother for all the support, patience and for believing in me even when I didn't.

And last, but not least, I would like to thank my friends for always being there for me, for better and for worse. You know who you are.

Abstract

One of the promising strategies concerning molecular therapy is gene therapy. By delivering genes for therapeutic purposes, we may treat both acquired and inherited genetic diseases. Non-viral vectors appear as a safer method of gene delivery into the cell, although not as efficient as viral vectors. Cationic lipids are an example of non-viral methods that have been thoroughly researched in the past few years as an effective tool to promote effective transfection of genetic material.

This project was focused on the development and characterization of lipoplexes based on dioctadecyldimethylammonium bromide/ chloride (DODAB/C): 1-monooleoyl-rac-glycerol (MO) liposomes with the inclusion of a cholesterol derivative 3β [N-(N',N'- dimethylaminoethane)carbamoyl cholesterol (DC-Chol) for transfection. Monoolein (MO) is a neutral lipid that seems to function well as a helper lipid since it affects the physicochemical properties of the lipoplexes and interferes with lipoplex-cell interactions. The physicochemical characteristics of DODAB/MO cationic liposomes were previously studied and their transfection capacity evaluated (Neves Silva *et al*, 2012). The results show a successful mediation of *in vitro* cell transfection by the DODAB:MO formulations. DC-Chol is a synthetic cationic molecule derivate from cholesterol. It has been used before in lipoplex assemblies in order to enhance transfection efficiency.

The results in this project show that the counterion exchange (bromide and chloride) has a significant effect in terms of particle size and Zeta potential. Furthermore, the liposome preparation method affects the physico-chemical properties of the particles, as well as the citotoxixity and transfection efficiency. The inclusion of DC-Chol has different effects on transfection efficiency and citotoxicity when included in DODAB:MO or DODAC:MO lipoplexes, depending on the molar ratio and on the preparation method of the liposomes.

Overall, DODAC:MO:DC-Chol lipoplexes arise as promising non-viral vectors for transfection.

Resumo

A terapia génica é uma das estratégias promissoras no que diz respeito à terapia molecular. Ao entregar às células e tecidos genes para fins terapêuticos, tanto doenças genéticas herdadas como adquiridas podem ser tratadas. Os vectores não virais aparecem como um método mais seguro de entrega de genes no interior das células, apesar de não serem tão eficazes como os vectores virais. Os lípidos catiónicos são um exemplo de métodos não virais que têm sido exaustivamente investigados nos últimos anos como meio capaz de realizar uma transfeção eficaz do material genético.

Neste projecto, focou-se no desenvolvimento e caracterização de lipoplexos baseados em lipossomas catiónicos de Brometo/Cloreto de Dioctadecildimetilamónio (DODAB/C): Monooleína (MO) e com a inclusão um derivado de colesterol (DC-Chol), a fim de testar a sua eficácia de transfecão. A monooleína (MO) é um lípido neutro que parece funcionar bem como lípido auxiliar, uma vez que afeta as propriedades físico-químicas dos lipoplexos e interfere com as interações lipoplexo-célula. As características físico-químicas dos lipossomas catiónicos DODAB:MO foram previamente estudadas e a capacidade de promover transfecção de lipoplexos preparados a partir destes lipossomas foi demonstrada (Neves Silva *et al.*, 2012). Os resultados deste estudo revelaram sucesso na transfeção *in vitro* de células mediada pelas formulações testadas de DODAB:MO. O DC-Chol é uma molécula catiónica sintética derivada do colesterol. Esta molécula é muitas vezes utilizada em composições liposómicas utilizadas para produzir lipoplexos a fim de aumentar a eficiência de transfeção dos mesmos.

Os resultados mostram que a troca de contra ião (brometo e cloreto) tem um efeito significativo em termos de tamanho médio e do potêncial ζ das partículas. Além disso, o método de preparação dos lipossomas também afecta as propriedades físico-químicas das partículas, bem como a sua eficiência de transfeção e citotoxicidade. A inclusão de DC-Chol tem efeitos diferentes sobre a citotoxicidade e a eficiência de transfeção quando incluídos em lipoplexos DODAB:MO e DODAC:MO, dependendo da fração molar incluída e do método de preparação dos lipossomas.

Em geral, os lipoplexos DODAC:MO:DC-Chol surgem como promissores vetores não virais para transfeção *in vitro.*

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Abbreviations

A	Adenine
C	Cytocine
DC-Chol	3β [N-(N',N'- dimethylaminoethane)carbamoyl cholesterol
DLS	Dynamic Light Scattering
DMEM	Dulbecco's Modified Eagle's medium
DNA	Deoxyribonucleic acid
DODAB	Dioctadecyldimethylammonium bromide
DODAC	Dioctadecyldimethylammonium chloride
DOPC	Dioleoyl-phosphatidyl-choline
DOPE	Dioleyl-phosphatidyl-ethanolamine
DOTAP	1,2-dioleoyl-3-trimethylammonium-propane
DOTMA	N [1-(2,3dioleyloxy) propyl]- N,N,N-trimethylammonium chloride
FBS	Fetal Bovine Serum
FRET	Fluorescence Resonance Energy Transfer
G	Guanine
H^{C}_{I}	Micellar hexagonal structure
H^{C}_{II}	Hexagonal structure
L^{C}_{α}	Lammelar structure
LDH	Lactate dehydrogenase
LUV	Large Unilamellar Vesicle
L_{α}	Lammelar disordered fluid state
L_{β}	Lammelar ordered gel state

MLV	Multilamellar Vesicle
MO	1-monooleoyl-rac-glycerol (Monoolein)
MTT	(3,(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide))
NADH	Nicotinamide adenine dinucleotide
PDI	Polidispersity index
pDNA	Plasmid DNA
PS	Phosphatidylserine
Rho PE	Rhodamine-DHPE
siRNA	Short-interfering RNA
SUV	Small Unilamellar Vesicle
T	Thymine
T_m	Transition temperature
β-gal	β-galactosidase

I. Introduction

1. Gene therapy

The concept of gene therapy has evolved in the last few years. At first, this new technology seemed a revolutionary promise to cure almost any disease to which the genetic or molecular basis was understood. Primarily, gene therapy's main goal was replacing a deficient gene in a genetically inherited disease with a normal copy of that gene. This goal was soon extended to acquired diseases. A more actual approach offers a wider perspective by referring to the potential use of nucleic acids such as siRNAs, plasmid DNA or antisense oligonucleotides in order to modulate gene function for therapeutic purposes (Hoag, 2005; Wasungu, 2006).

1.1 Viral and non viral methods

Concerning the vectors used for nucleic acid transfer, we can roughly divide vectors into two categories: viral and non-viral methods.

Viral methods are based on the construction of viral particles containing the gene of interest within the viral genome. The viral vectors used include Retrovirus, Adenovirus and Lentivirus. These viral particles lack pathogenic functions and are incapable of self-replication. However, recombination events may occur and generate an infection-competent virus (Wasungu, 2006). Additional hazards include innate mutational issues, particularly with adenovirus, mutational insertion risks, mostly derived from retrovirus and potential appearance of oncogenicity (Thomas *et al*., 2003). These hazards have seriously limited the clinical usefulness of this gene-delivery method (Glover *et al*., 2005). Clinical trials like the one reported by Raper and colleagues (Raper *et al*., 2003) where a patient with partial ornithine transcarbmaylase (OTC) deficiency submitted to adenoviral gene transfer died of systemic inflammatory reaction confirm the safety issues inherent to viral methods.

Non-viral methods involve the delivery of DNA containing the gene of interest by means of physical or chemical techniques. These delivery systems rise as a safer alternative to viral methods as they are immunologically inert, easier to produce, and can accommodate larger molecules and wider variety of cargo then the viral systems,

although the efficiency of transfection is lower (Glover *et al.,* 2005). There is a variety of nonviral delivery systems that have been developed for gene therapy in different clinical settings. As examples of conventional non-viral methods for gene delivery, we have the physical methods like DNA microinjection into cells, electroporation and ballistic delivery, which promote the entry of nucleic acid into the cells by disrupting the plasma membrane with electric pulses and bombardment of DNA-coated metal particles at high velocity respectively (Glover *et al.,* 2005). There are also cationic polymer based methods, like cationic polymer-based gene delivery systems (polyplexes) and DNA/Cationic Liposomes complexes (lipoplexes) (Glover *et al.,* 2005; Li and Huang, 2000; Niidome and Huang, 2002; Templeton, 2001). This study will focus on lipoplexes as a nonviral gene delivery system.

1.2 DNA (deoxyribonucleic acid)

The DNA molecule carries the genetic information on which human and other life forms are built. Therefore, it makes all sense that the DNA molecule is used in the development of a group of therapeutics modeled on its endogenous structure.

Briefly, DNA has a three-dimensional structure consisting in a double helix formed by two helical strands which are coiled around a common axis (Figure 1A). Each strand is composed by nucleotides (also called bases), monomers which consist of a purine or pyrimidine base, a sugar (Deoxyribose in the case of DNA) (Figure 1B) and a phosphate group. DNA is composed by four different bases: thymine (T), guanine (G), adenine (A) and cytosine (C) (Figure 1C) The bases on one strand unite to the bases of the opposite strand internally via hydrogen bonds between the purine and pyrimidine bases, linking the two strands together (Figure 1A)(Lodish *et al.*, 1996).

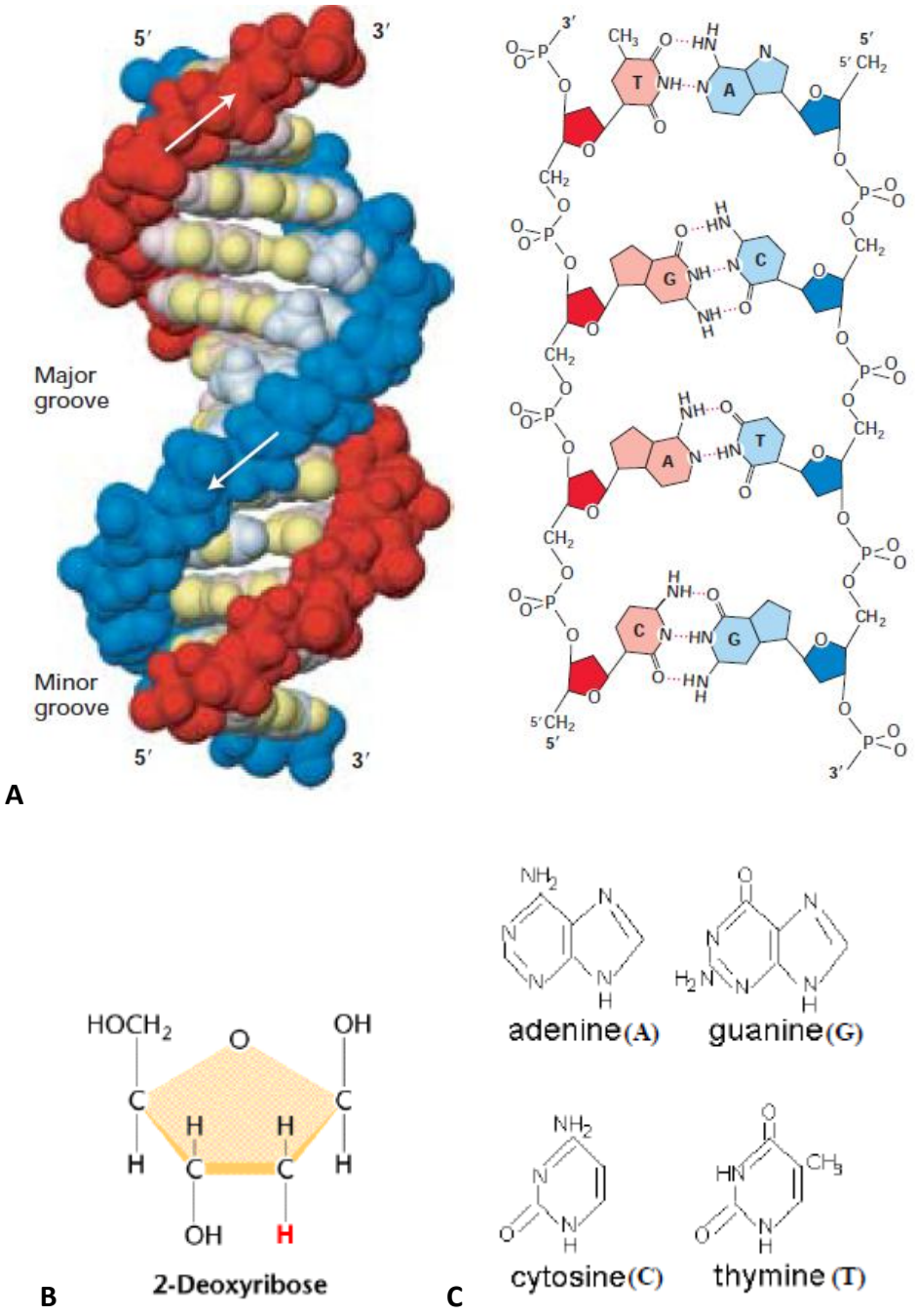


Figure 1. (A) DNA structure; (B) Deoxyribose present in DNA; (C) nucleotides that compose DNA (Lodish *et al*., 1996).

1.2.1 Plasmid DNA

Plasmids are high molecular weight, circular, double stranded DNA molecules which are distinct from chromosomal DNA. They occur naturally in bacteria and in some lower eukaryotic cells, like yeasts, and are duplicated before every cell division,

the same as the host's chromosomal DNA. Different types of plasmids can be constructed *in vitro* and often used as vectors for DNA cloning (Stryer, 1996).

In a typical plasmid we can find a genome with promoter regions which are critical for the transcription process, a multiple cloning site where DNA fragments may be inserted to the plasmid and at least a gene expressing antibiotic resistance (Figure 2).

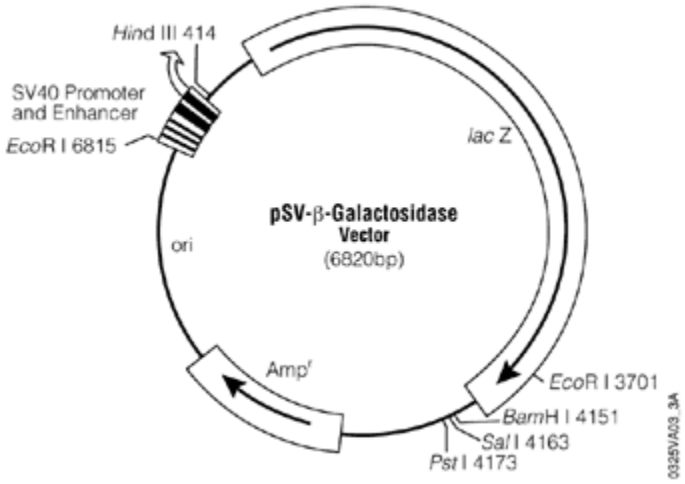


Figure 2. Map of the plasmid DNA used in this thesis (pSV-β-Galactosidase Vector).

Plasmids can easily be isolated and purified after being grown in bacteria. This may be done by transferring the plasmid into bacteria and allowing the bacteria to grow overnight, followed by bacteria lysis.

The application of plasmid DNA in gene therapy involves the introduction of a transgene into the cells in order to correct genetic errors inherent to the production of incompetent copies of a given protein or even to the lack of protein production altogether. The efficiency of this treatment depends on the access gained by the plasmid DNA to the nucleus through the nuclear pores, once it entries the cytoplasm. Besides disease treatment, plasmids may be applied to function as DNA vaccines or even in suicide gene therapy. So, on a molecular level, the plasmid DNA molecules may be considered as pro-drugs (Patil *et al*., 2005; Uherek and Wells, 2000).

2. Gene delivery

Some conditions are vital for a successful gene delivery. These conditions are the condensation of the DNA and its protection against intracellular nucleases, lipoplex adhesion onto the cell surface, lipoplex internalization followed by fusion with the endosome membrane, DNA escape from the endosome and, finally, DNA entry into the nucleus and its expression (Hui *et al*., 1996; Zhdanov *et al.*, 2002).

2.1 Transfection mediated by lipoplexes

2.1.1 Cellular binding

Lipoplexes usually bind to the cellular surface mainly as a result of nonspecific ionic interactions between negative charges on the membrane surface and positive charges of the lipoplexes. There may also be specific targeting ligands interactions when these are incorporated in the system (Khalil *et al.*, 2006). The presence of serum proteins, present in the culture medium, can interfere with the interaction between cells and lipoplexes, decreasing the efficiency of the transfection. It seems that the extent of interference of the serum components with the transfection efficiency is related to the cationic lipid chemical structure as well as the liposomal formulation and charge ratio (+/-) of the lipoplexes (Zelphati *et al*., 1998). Nevertheless there is still incomplete knowledge on the lipoplex-serum interaction.

2.1.2 Endocytosis

Given the general cellular membrane structure, the presence of a specific ligand as well as the vectors overall charge content will influence the way lipoplexes interact with the membrane (Chestnoy and Huang, 2000). There are two main pathways by which DNA-Lipid complex may enter the cell. One way is by endocytosis followed by the destruction of an endosome inside the cell and the other way is by direct fusion of the lipoplex with the cellular membrane (Figure 3). Endocytosis is the major way by which most complexes enter the cell and only a small percentage is internalized by direct fusion with the cell membrane (Hoekstra *et al*., 2007; Khalil *et al*., 2006). It

seems that lipoplexes may enter through clathrin-mediated endocytosis or caveolae-mediated endocytosis (Hoekstra *et al*., 2007). For example, Rejman (Rejman *et al*.,2005) studied the effect of inhibitors of clathrin-mediated endocytosis (chloropromazine and K^+ depletion) and of caveolae-mediated endocytosis (filipin and genistein) on A549 pneumocytes and HeLa cells of FITC–poly-L-lysine-labeled DOTAP/DNA lipoplexes and on their transfection efficiency with the luciferase gene, concluding that the lipoplex uptake occurs only by clathrin-mediated endocytocis and transfection efficiency was entirely abolished by blocking clathrin-mediated endocytocis, whereas no effect was observed with the inhibition of the caveolae pathway. It seems important in these kinds of studies to establish a direct correlation between the pathway of entry and the transfection efficiency so that one can deduce how the pathway of entry may relatively contribute to a good transfection activity.

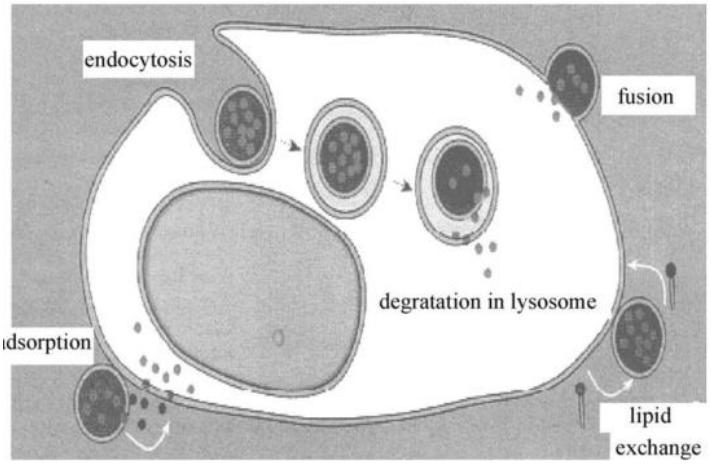


Figure 3. Proposed mechanisms of lipoplex entry into the cell (Zhdanov *et al.,* 2002).

Once inside the endosome, it is important that plasmid DNA escapes to the cytosol before reaching the lysosomes to avoid DNA degradation (Hoekstra *et al*., 2007).

The size of the lipoplexes is an important aspect to consider in determining the nature of the entry pathway by endocytosis. The nature of the entry pathway and its efficiency can also be cell type dependent, according to the endocytic machinery of the

cell. For instance, Rejman (Rejman *et al.,* 2004) studied the effect of cholesterol depletion and inhibitors on the internalization of fluorescent latex beads of defined various sizes, ranging from 50nm to 1000nm, by non-phagocytic B16 cells. The study revealed that the size of the particles strongly influenced the mechanism by which they were internalized as well as their subsequent intracellular route. Internalization of particles with sizes up to 200nm involved clathrin-coated pits. For particles with 500nm, the caveolae-mediated internalization becomes the predominant pathway of entry.

Still, the relative contribution of either pathway in lipoplex internalization and their relative contribution to the transfection efficiency of lipoplexes need a better insight (Zuhorn *et al.,* 2007).

2.1.3 Endosomal escape

Escape from the (early) endosome into the cytosol is a critical step in order to avoid plasmid DNA degradation in the lysosome. Thus, it also constitutes one of the main criteria to successful transfection efficiency (Hoekstra *et al.,* 2007). The escape into the cytosol by adenovirus involves the lysis of the endosomal membrane structure, and the hexagonal structure of the lipoplexes is thought to play a parallel role with the adenovirus mechanism to transfect cells (Wasungu and Hoekstra, 2006), although naturally, the lipolexes lack the protein machinery to promote the endosomal membrane destabilization.

A mechanism of endosomal membrane destabilization involving lipoplex-induced processes seems to be promoted by non-lamellar phases, including the hexagonal H_{II} and H_I or a cubic phase. Apparently, lipoplexes that adopt these structures strongly promote transfection, and this fact is consistent with this mechanism. The inclusion of PEGylated lipid derivatives, for example, in the DNA/Cationic lipid complexes, appears to have a stabilizing effect on the membrane bilayer structure and, at the same time, an inhibition effect on DNA dissociation. Moreover, the phospholipid phosphatidylserine (PS), present in the outer leaflet of the endosomal membrane seems to play an important role in this process as it facilitates the lamellar to non-

lamellar hexagonal-phase transition of the lipoplexes and thus, releasing the DNA from the lipoplex (Hoekstra *et al*., 2007; Wasungu and Hoekstra, 2006).

Then again, there may be a translocation of the cationic lipids into the endossomal membrane by the interaction of non-lamellar intermediates followed by a local increase which may give rise to pore formation of transient stability and membrane destabilization. The rate of lateral diffusion of the surrounding membrane lipids determines this. And so, the DNA may gain access to the cytosol (Hoekstra *et al*., 2007). It is still unclear if DNA dissociation occurs before or simultaneously with the perturbation of the endosomal membrane. Furthermore, it is also unclear if the complete lysis of the endosome is necessary for the DNA transfer or if it may occur across the perturbed membrane (Hoekstra *et al*., 2007).

New insights on molecular mechanisms underlying lipoplex induced endosomal membrane destabilization can be given by modeled endosomal membranes for instance. Berezhna and coleagues (Berezhna *et al*., 2005) performed a study using GUVs as model endosomal membranes in order to investigate what part some individual phospholipids play in interaction with lipoplexes, using laser scanning imaging in conjunction with fluorescence cross-correlation analysis.

Although there has been considerable progress in recent years in the study and knowledge of the barriers in lipoplex mediated transfection, there's still a long way to go. Further studies are needed to understand the relative contribution of endocytosis in terms of effectiveness of internalization, efficiency of gene escape and, eventually, transfection efficiency, since this is the major pathway of entry of lipoplexes.

2.1.4 Nuclear Delivery

The final barrier to lipofection, and probably the most challenging too, is the nuclear membrane. Indeed, when plasmid DNA encoding β-galactosidase is injected directly into the nucleus, the gene expression was much higher when compared with when the same plasmid was injected into the cytosol. Furthermore, when DNA complexed with cationic lipids is directly injected into the nucleus, the complexed DNA is not expressed, suggesting that DNA has to be released from the lipoplex somewhere in the cytoplasm (Pollard *et al*., 1998).

During mitosis, the nuclear membrane is fragmented and this would allow the plasmid DNA into the nucleus. This seems to be the most widely accepted explanation as to how the DNA is delivered to the nucleus, so cell division plays an important role in the nuclear translocation of transgenes.This fact, nevertheless, cannot be the only explanation, since nondividing cells are targeted in *in vivo* transfection (Khalil *et al*., 2006).

3. Lipids

Lipids can have various usages in biological systems which make them important molecules for living beings. Some lipids assemble themselves into bilayer lipid membranes that separate cellular compartments from the external environment and participate in cellular regulation by mediating signal transduction processes. The lipid molecules found in the membrane are amphipathic molecules, which signifies they have a hydrophilic tail oriented to the inner part of the bilayer and a hydrophobic head oriented to the aqueous environment.

The model proposed by Singer and Nicholson (1972), called the "fluid mosaic" (Figure 4) proposes that biological membranes are composed of lipids, proteins and carbohydrates and describe that the nature of the lipid bilayer allows for considerable molecular motion in the lipid matrix and provides an appropriate milieu for the function of the proteins found in the membrane. (Alberts *et al.,* 1994)

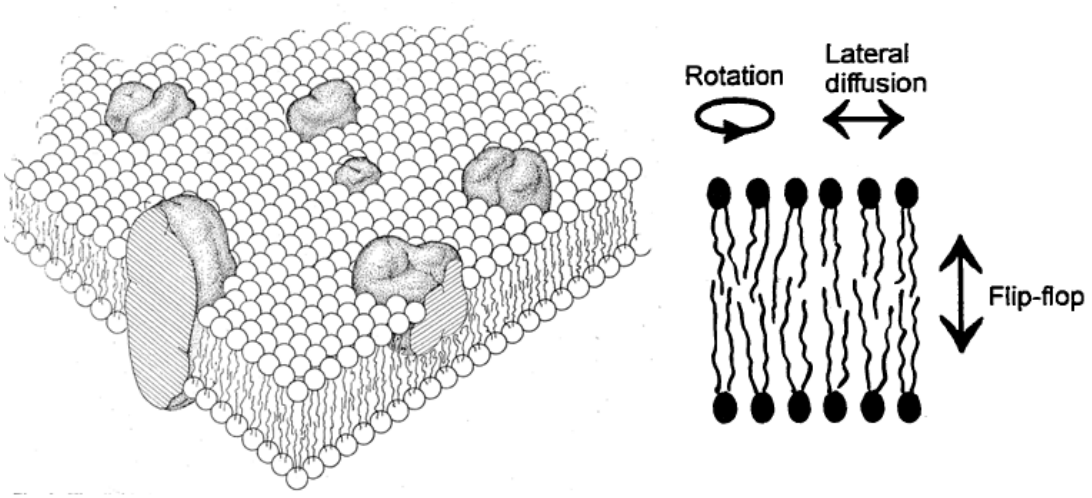


Figure 4. Cellular membrane according to the "fluid mosaic" model and phospholipid mobility within the bilayer (adapted from Singer and Nicolson, 1972).

Depending on the structure and function, lipids can be classified into different groups. Among the lipids that constitute biological membranes we can find three groups: glycerophospholipids, sphingolipids, and sterols. (Chang, 2005)

The surrounding environment is a very important factor in the structural behavior of lipids. Lipids may adopt different conformations when hydrated and the propensity of these molecules to adopt different phases as a response to some external variables like pH, presence of ions or temperature is referred to as lipid polymorphism. These phases include the micellar, the lamellar, the cubic and the inverted hexagonal phases (Figure 5).

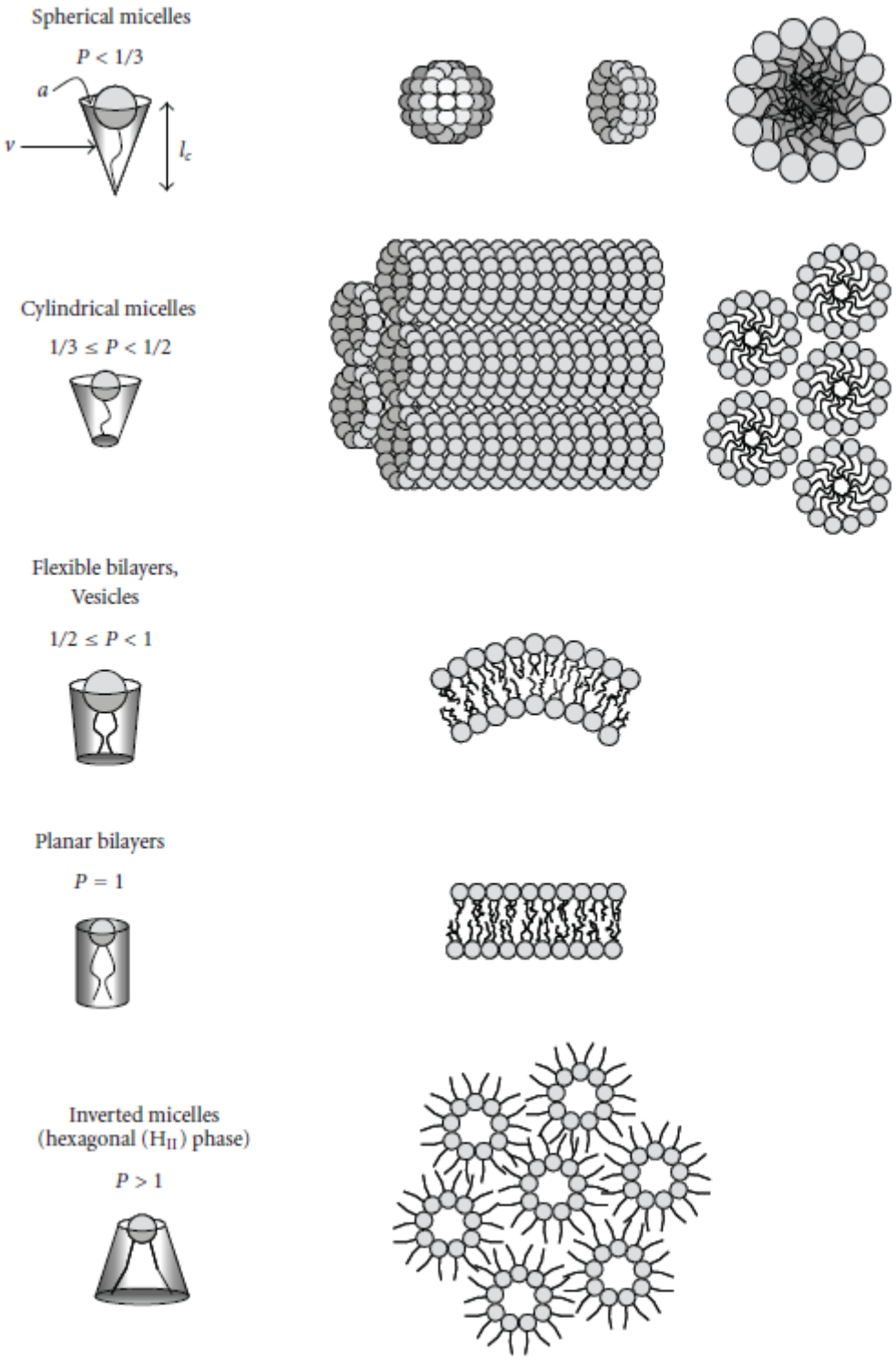


Figure 5. Impact of packing parameter *p* on lipid assemblies formed in aqueous solutions (Balazs and Godbey, 2011).

The packing parameter allows to predict which structure the molecules will adopt (Balazs and Godbey, 2011). This packing parameter is given by:

$$P = \frac{v}{al}$$

And is defined as the ratio of the hydrocarbon volume, *v*, and the product of the effective head group area, a , and the critical length of the lipid tail*, l* . It is also related to the curvature by:

$$\frac{v}{al} = 1 + Hl + \frac{Kl^2}{3}$$

Where H is the mean curvature and K is the Gaussian curvature (Antonielli and Förste, 2003).

When p < 1/3 the molecules adopt preferentially a conical shape, forming conventional spherical micelles. When 1/3 < p < 1/2, the molecules adopt a geometry resembling a truncated cone, forming non-spherical micelles. The lipidic bilayer is formed when 1/2 < p < 1, and the molecules adopt a nearly cylindrical shape. At last, when p > 1, we can see the formation inverted structures with negative spontaneous curvature (Antonielli and Förster, 2003; Balazs and Godbey, 2011; Ryhänen, 2006).

Lipids can also exist in “gel” state (L_β) or in a fluid “liquid crystalline” state (L_α) depending on the temperature (Figure 6). The transition temperature (T_m) is specific for each lipid. The lipids arranging in a gel state, bellow the transition temperature, are rigid and in order. This is due to the carbon-carbon bonds of the acyl chains, which tend to extend into an all-*trans* conformation. When they reach the L_α state, the acyl chains become shorter, reaching an all-*gauche* conformation and the packing parameter increases between individual lipid molecules (Mok, 1998), also increasing their diffusion. The lipid bylayer becomes more fluid and elastic in this state (Ryhänen, 2006).

The transition from gel to fluid state, affects the properties and structure of lipid bilayer at the level of lipid acyl chains, individual lipid molecules and at the supramolecular assembly as a whole (Ryhänen, 2006).

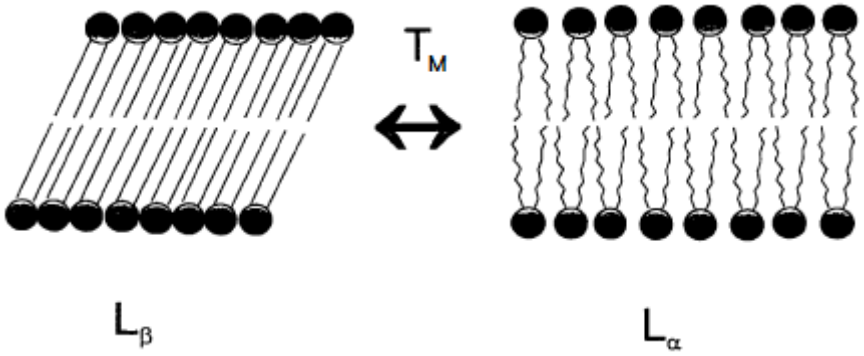


Figure 6. Lipid bilayer and its transition from gel" state (L_β) into fluid "liquid crystalline" state (L_α) (adapted from Mok, 1998).

4. Liposomes

Liposomes are good models of biological membrane systems to study physical properties and functional roles of lipid components. As non-viral vectors, they have unique advantages like having diverse morphologies and compositions, different release characteristics, lack of immunogenic response, and a low cost production (Balazs and Godbey, 2011). They may contain more than one lipid bilayers and so, we may classify them into three types: Multilamellar Vesicles (MLVs), Large Unilamellar Vesicles (LUVs) and Small Unilamellar Vesicles (SUVs) (Figure 7)

MLVs can easily be formed upon a lipid film hydration and are usually composed of concentric layers of bilayers. The size of this vesicles ranges from 0.5-10 nm, so they are very heterogeneous in size (Mayer *et al*., 1986). MLVs are not widely used for drug delivery since they show low trapping volumes. Furthermore their large sizes lead to a rapid clearance. However, they can be very useful for studying the structural and motional properties of lipids.

LUVs are vesicles possessing a single bilayer with sizes ranging from 50 to 200 nm. They can be formed by several methods but the most convenient is extrusion through polycarbonate filters with a well defined size pore (Mayer *et al.*, 1986). They

show longer circulation lifetimes in vivo due to their smaller size relatively to de MLVs, so this makes them a frequently used model for drug delivery.

SUVs are vesicles composed of a single bilayer with sizes ranging from about 25 to 50 nm in diameter (Mok, 1998) and can be produced by submitting MLVs to sonication (Huang, 1969).

A method of liposome preparation proposed in order to optimize the properties of lipid-DNA complexes consists in both extrusion of the liposomes, so as to obtain LUVs, and controlled mixing of lipid and DNA. The results seem to show that the lipoplexes exhibit a narrow distribution and small sizes, which might be adequate for their *in vivo* application (Pedroso de Lima *et al*., 2001).

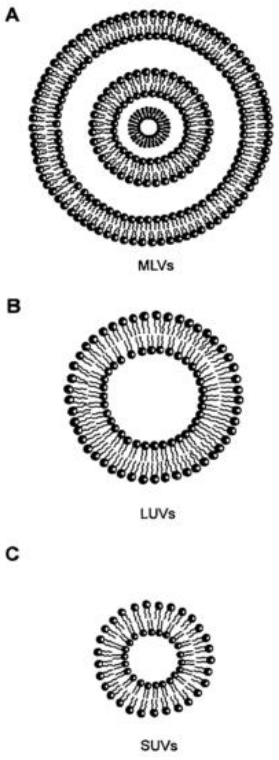


Figure 7. Multilamellar Vesicles (MLVs), Large Unilamellar Vesicles (LUVs) and Small Unilamellar Vesicles (SUVs) (adapted from Mok, 1998).

4.1 Cationic lipids and liposomes

There are three parts composing cationic lipids: a hydrophobic anchor, a linker, and a head group (Figure 8).

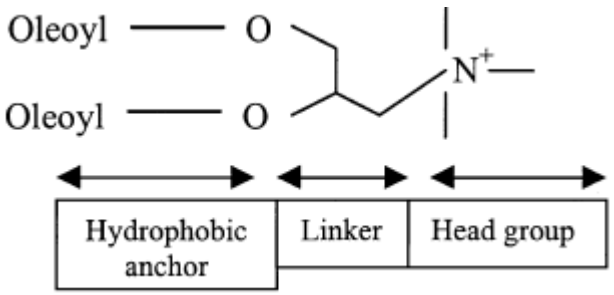


Figure 8. Scheme exemplifying a cationic lipid (Chestnoy and Huang, 2000).

The hydrophobic anchors can be grouped into two major types of hydrophobic moieties: aliphatic chains and cholesterol-based derivates. Traditionally, double-tailed cationic lipids are more efficient and less toxic than the single tailed cationic lipids (Hongtao *et al*., 2006). So, the majority of synthesized cationic lipids have double chain hydrocarbons. Commonly, they are capable of forming liposomes by themselves but it is usual to use another phospholipid as helper in the formulations for cationic lipid transfections (Chestnoy and Huang, 2000). The length of the alkyl chain in transfection activity can also influence the transfection activity (Felgner *et al*., 1994).

The linker, as the name states, represents any chemical part between the head group and the hydrophobic anchor. This link also plays an important role in gene transfer as stated, for example, by Liu (Liu *et al*., 1997), which concluded that less stable ester bonds are not as beneficial as stable ether bonds for in vivo gene transfer.

As to the head group, cationic lipids may be monovalent or multivalent, according to the number of charges. In monovalent lipids, we can find a head group consisting of either tertiary or quaternary ammonium groups (Chestnoy and Huang, 2000). Chemical modifications were also assessed by Felgner (Felgner *et al*., 1994).

The cationic nature of the lipid is a determinant factor to its toxicity (Hongtao *et al.*, 2006). It is important to state that the definition of a good cationic lipid for gene

transfer is not a solid one, since their efficiency for *in vitro* experiments in not necessarily the same as for *in vivo* experiments (Chestnoy and Huang, 2000), and further studies must be performed.

Cationic liposomes may be formed from a single cationic lipid or, as generally happens, from a combination of a cationic lipid and a neutral lipid. They were first introduced by Felgner (Felgner *et al.,* 1987) as DNA transfection agents when Felgner and colleagues successfully transfected the COS-7 cell line with complexes between DNA and 2,3-dioleyloxypropyl-1-trimethyl ammonium chloride (DOTMA) cationic liposomes. Since then, new cationic liposome formulations have been reported to successfully transfect the cells and some of them have even been commercialized and engineered for a wide range of applications (Samad *et al*., 2007).

Transfection efficiency varies according to many factors, including the type of cationic lipid used or the cell type that is transfected. 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) , N [1-(2,3dioleyloxy) propyl]- N,N,N-trimethylammonium chloride (DOTMA) (Felgner *et al.*, 1987), dioctadecyldimetylammonium bromide (DODAB), or synthesized derivatives from biologically active compounds including the cationic cholesterol derivatives such as 3β [N-(N',N'- dimethylaminoethane)carbamoyl cholesterol (DC-CHOL) are some examples of the cationic lipids used for transfection. The addiction of a "helper" lipid seems to enhance transfection efficiency, although the mechanism responsible for such effect is not fully understood. The inclusion of neutral lipids in the liposome formulation facilitates the complex fusion to the cellular membrane because they tend to form nonbilayer structures which are related to membrane fusion intermediates (Hui *et al*., 1996). Dioleyl phosphatidylethanolamine (DOPE), cholesterol and dioleoyl phosphatidyl choline (DOPC) are three neutral lipids often incorporated in liposomal formulations. The DOPE- containing liposomes, as well as liposomes with some galactosydated cholesterol derivates seem to achieve high transfection efficiencies regarding human hepatoma cells, Hep 2. They also seem to exhibit low toxicity (Zhang *et al*., 2004).

In this thesis, various formulations of liposomes composed of Dioctadecyldimethylammonium bromide (DODAB), Dioctadecyldimethylammonium

chloride (DODAC), 1-monooleoyl-rac-glycerol (MO) and 3β [N-(N',N'-dimethylaminoethane)carbamoyl cholesterol (DC-Chol) are studied.

Dioctadecyldimethylammonium bromide (DODAB) was first synthesized by Kunitake and Okahata (Kunitake and Okahata, 1977), who prepared and characterized small unilamellar DODAB vesicles. Since then, Dioctadecyldimethylammonium bromide and chloride (DODAB/C) have been prepared through a series of methods in order to obtain size-controlled structures with long-term stability for different applications, namely as DNA carrier vehicles. They are synthetic lipids that tend to form LUV's in the presence of excess water. These vesicles have their structural organization dependent on lipid concentration, the solvent composition, the methods of preparation and the temperature. They are also influenced by the presence of other substances (Feitosa *et al.*, 2009; Neves Silva *et al.*, 2009).

Monoolein, 1-monooleoyl-rac-glycerol, is an amphiphilic neutral lipid of natural origin. This molecule has the particularity of, even in the presence of excess water, presenting two inverted bicontinuous cubic phases (Oliveira *et al*., 2012). MO has a fluidizing effect that seems to contribute to the complexation efficiency of pDNA in MO-based lipoplexes in a favorable way (Neves Silva *et al*., 2008; Neves Silva *et al*., 2011; Real Oliveira *et al.,* 2010; Real Oliveira *et al.,* 2011).

Cholesterol is a neutral lipid and a major component of biological membranes in most eukaryotic cells. Cholesterol may act as a "disordering agent" or as an "ordering agent". At high concentration, it can form cholesterol rich domains within the lipid bilayer and also condensed complexes or cholesterol crystallites with phospholipids (Hungerford *et al*., 2005; Hungerford *et al*., 2006). It is frequently used in liposome formulations due to its biocompatibility and the stability it confers to lipid membranes (Balazs and Godbey, 2011). DC-Chol was first synthesized by Gao and Huang (Gao and Huang, 1991). It contains a cholesterol moiety attached by an ester bond to a hydrolysable dimethylethylenediamine. DC-Chol was found to have reduced toxicity when compared to Lipofectin in some cell lines (Gao and Huang, 1991). It is no surprise then, that DC-Chol and other cholesterol derivates have been incorporated in lipoplex assembly in order to increase transfection (Balazs and Godbey, 2011; Bennet *et al.,* 1995).

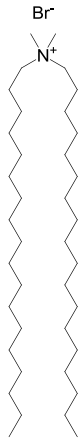
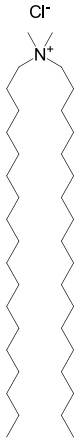
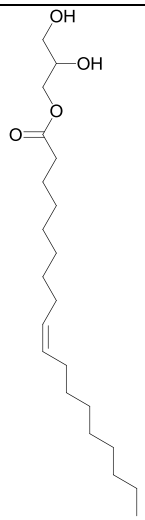
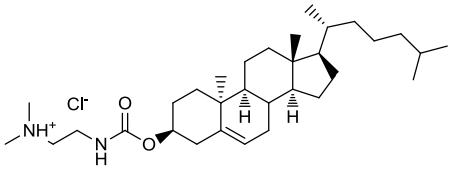
DODAB	*DODAC*	*MO*	*DC-Chol*
			
$C_{38}H_{80}BrN$	$C_{38}H_{80}ClN$	$C_{21}H_{40}O_4$	$C_{32}H_{57}ClN_2O_2$

Table 1. Structure of the lipids relevant to this study

The DODAB:MO system has been previously produced and optimized in order to promote transfection in vitro of human cell lines (Neves Silva *et al*., 2011). The exchange of the counter ion in DODAC (Chloride instead of Bromide) might have an effect in the colloidal characteristics of the system and affect it's stability in the presence of salt and serum. Also, the inclusion of DC-Chol in the system might have an effect on the membranes fluidity, molding it, which should influence the systems complexation with the plasmid DNA and, furthermore, its release inside the cell.

5. Lipoplexes

Both DNA and cationic liposomes suffer structural changes during the complexation process (Zhang *et al.*, 2003).

Cationic lipids interact with negatively charged DNA molecules, which results in the formation of DNA/Cationic liposome complexes (lipoplexes). In the complex formation, DNA electrostatically binds to the liposome surface and the two components rearrange themselves into a new structure. The way lipoplexes interact

with the membrane will be influenced by aspects like the presence of specific ligands and the vectors overall charge (Chestnoy and Huang, 2000). But the main reaction source comes from the positive charge on lipoplex surface which reacts spontaneously with the cellular membrane (Zhdanov *et al*, 2002).

The study and characterization of the lipid-DNA complexes is very important since a better understanding of the assembly of these complexes might help to establish a correlation to their biological activity. It is known that the transfection efficiency is highly dependent on the structure and properties of a given lipid-DNA complex (Chestnoy and Huang, 2000). Indeed Sternberg and colleagues (Sternberg *et al.*, 1994), using freeze-fracture electron microscopy, observed different kinds of structures of lipoplexes formed between plasmid DNA and cationic liposomes composed of DC-Chol and DOPE, depending on the DNA concentration and incubation time of the complexes. Experiments performed by Zhang (Zhang *et al*., 2003) using fluorescence resonance energy transfer (FRET) also suggested that the incubation time and charge ratio, among other experimental parameters, should be carefully controlled in order to achieve optimized transfection activity.

In the cationic lipoplex structures, DNA might be adsorbed onto the surface of the liposome or it might be surrounded by a lipid envelop (Ma *et al*., 2007). Usually the charge ratio (+/−) of the assembled complexes surpasses a value of 1. So we have complexes with a positive net charge which promotes an efficient binding with negative charges in the cell surface by electrostatic interactions (Hoekstra *et al*., 2007). The fact is, in different lipid mixtures, there has been observed several lipoplex morphologies. It has been proposed that some of these structures may correspond to metastable intermediates, spaghetti-like structure consisting of double-stranded DNA wrapped around the cationic and helper lipids. A lamellar structure, L^{C}_{α}, and hexagonal structure, H^{C}_{II}, (Figure 9) are the two symmetries identified as equilibrium ordered phases (May *et al*., 2000).

The L^{C}_{α} consists of DNA monolayers “sandwiched” between stacked lipid monolayers and with intervening water gaps (Figure 9A). The electrostatic attraction between the cationic lipid bilayer and the negatively charged DNA confer stability to this morphology. Accordingly, the H^{C}_{II} phase may be considered as an ordinary inverse-hexagonal lipid phase, in which the DNA can be found intercalated within the water

tubes (Figure 9B) (May *et al.,* 2000). It has been reports as well of a micellar hexagonal phase, H^C_I (Figure 9C). In this phase the DNA can be found in the interstices of the lipid micelle arrangement (Tresset, 2009).

The concept of lipoplex formation is relatively simple, however, this process can determine the morphology and transfection potential of these structures (Ma *et a*l, 2007).

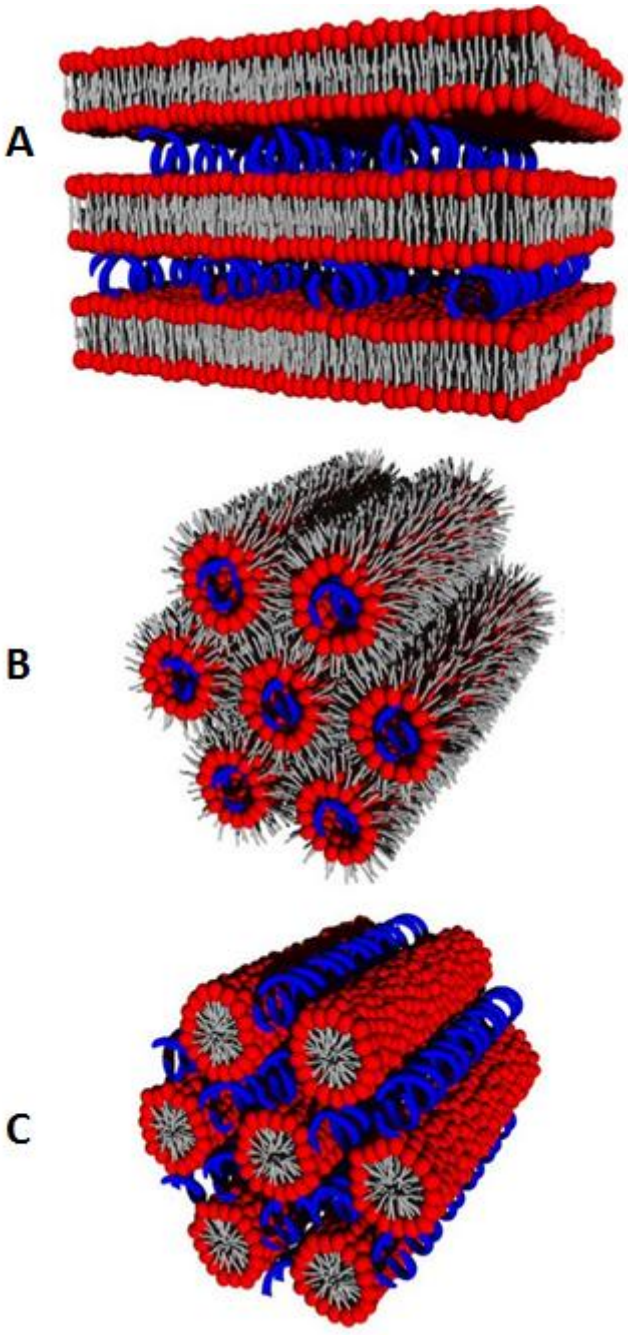


Figure 9. Lipoplex structures. (A) Lamellar phase (L^C_α); (B) Inverted hexagonal phase (H^C_{II}); (C) micellar hexagonal phase (H^C_I)(adapted from Tresset, 2009).

6. Outline of this study

Since their first introduction, cationic lipids have gained a widespread interest as a non-viral vector. But they are far from being perfect delivery vectors, and many issues are still to be overcome, hence the need for further investigation in this field.

Based on the DODAB:MO system, previously studied, this work aims to develop and characterize a new system, based on DODAC:MO:DC-Chol lipoplexes, suitable to perform efficient transfection.

On a first basis, the aim is to study how the the exchange of the Bromide counter ion for the Chloride affects the physical-chemical characteristics of the liposomes and the derived lipoplexes. It will be also studied the effect of inclusion of DC-Chol in the liposomal formulation. To do so, complexation studies using FRET technique, as well as measurements of size and zeta potential of the liposomes and lipoplexes were performed. The behavior of the system in the presence of serum and NaCl it was also investigated.

Secondly, the toxicity of the liposomes with different formulations and transfection efficiencies of this new system were assessed.

II. Materials and Methods

A) Materials

Dioctadecyldimethylammonium bromide (DODAB) and Dioctadecyldimethylammonium chloride (DODAC) were purchased from Tokyo Kase.

3ß-[N-(N',N'-dimethylaminoethane)-carbamoyl] cholesterol hydrochloride (DC-Chol) was purchased from Avanti Polaris Lipids.

Nucleopore Track-Etch Membranes were purchased from Whatman.

1-monooleoyl-rac-glycerol (MO), Dulbecco's Modified Eagle's Medium (DMEM), Antibiotic/Antimycotic solution, Bovine Serum Albumin (BSA), (NADH), *The Wizard Plus Midipreps DNA Purification System* and Bradford reagent were purchased from Sigma-Aldrich.

Heat-inactivated Fetal Bovine Serum (FBS), UltraPure™ Salmon Sperm DNA solution and rhodamine DHPE were purchased from Invitrogen (UK).

BOBO-1 was purchased from Molecular Probes (UK).

ß-Galactosidase Enzyme Assay System with Reporter Lysis Buffer was purchased from Promega (USA).

Opti-MEM I Reduced Serum Medium was purchased from Gibco (UK).

pSV-β-gal plasmid DNA was kindly offered by the Hematopoietic Biology Unit, Faculty of Medicine, University of Lisbon.

Escherichia coli Xl1-Blue competent cells were kindly given by the Microbiology laboratory in the Biology Department of the School of Sciences – University of Minho.

EzWay™ Transfection Reagent was kindly offered by Koma Biotech.

B) Methods

1. Preparation of liposomes

1.1 Ethanolic injection

One of the methods used to produce liposomes was ethanolic injection. Defined volumes taken from stock solutions of DODAB/C, MO, and DC-Chol (20mM) in

ethanol were dissolved in a know amount of ethanol (organic solvent) and injected in a volume of distilled water, preheated to 50ºC, under vigorous vortexing. The organic solvent evaporates when it comes into touch with the distilled water due to the heat. So we obtained an aqueous solution of liposomes DODAB/C:MO:DC-Chol (ratios 6:3:1, 5:4:1, 4:1:1) and DODAB/C:MO (2:1) at a final concentration of 1mM , mostly composed of multivesicular liposomes.

1.2 Film hydration method

Again, defined volumes taken from the lipids stock solution of DODAB/C, MO, and DC-Chol (20mM) in ethanol were placed in a rotary evaporator (Heidolph VV Micro Rotary Evaporator). The organic solvent was then evaporated under vacuum and at 50ºC, safely above the lipids phase transition temperature, until all traces of organic solvent were gone and a lipidic film was formed. The resulting lipidic film was then dispersed in a given volume of ultra-pure water in order to obtain a solution with liposomes DODAB/C:MO:DC-Chol (ratios 6:3:1, 5:4:1, 4:1:1) and DODAB/C:MO (ratios 2:1) with a final concentration of 1mM.

1.3 Extrusion

The solution containing the liposomes obtained from the film hydration method was placed in the extruder (Northern Lipids Lipex Extruder). Under a 4-8bar pressure, the liposomes were forced to pass trough polycarbonate filters (Whatman) with a defined pore size. In this case, the liposomes were submitted to one passage through a filter with a pore size of 400nm and then four passages through a filter with a pore size of 100nm. The mean diameter of the obtained population reflects the diameter of the used filter pore, and unilamellar vesicles were obtained. This process was carried out at 50ºC so that the lipids were in a fluid state and preventing, in this way, as much retention of lipid in the filters as possible.

2. Preparation of DNA solutions

2.1 Plasmid DNA

2.1.1 Transformation of competent cells

Plasmid DNA (pSV-β-Galactosidase control vector) was added to a 200µL aliquot of *Escherichia coli* Xl1-Blue competent cells and the mixture swirled and incubated on ice for 30 minutes. The mixture was heat-pulsed with occasional agitation at 42 ºC for 90 seconds, and placed on ice for 10 min. Then 800µL of SOC medium (2% tryptone peptone, 0.5% yeast extract, 2.5mM KCl, 10mM NaCl , 10mM $MgSO_4$, 10mM $MgCl_2$, 20mM glucose) was added and the tubes were incubated at 37ºC at 200 rpm, for 1 hour. The cells were then centrifuged at 14000 rpm for less than 10 seconds and the pellet resupended in 50µL of supernatant. The resuspended pellet was then pipetted to a petri dish with LB medium (1 % tryptone peptone, 0.5 % yeast extract, 1 % NaCl, 2 % agar) supplemented with ampicillin (100µg/µL) and spread gently and minimally using a bent (L-shaped) Pasteur pipette. A petri dish with competent cells not submitted to the transformation process was also used as control. These plates were incubated at 37ºC to establish colonies. Only transformed cells will form colonies since this strain is susceptible to ampicillin and the plasmid confers resistance to the cells. Once the colonies are grown, one of them is selected and transferred into 200mL of LB medium (1 % tryptone peptone, 0.5 % yeast extract, 1 % NaCl) at 37ºC at 200rpm overnight. This overnight recombinant *E. coli* culture is then harvested in order to isolate the plasmid DNA.

2.1.2 Purification of plasmid DNA

Wizard® Plus Midipreps DNA Purification System (Promega Madison, USA) was used to isolate the pSV-β-Galactosidase control vector from *Echerichia coli* Xl1-Blue. The isolated plasmid DNA was then ressuspended in ultra-pure water and measured with NanoDrop ND 100 Spectrophotometer in order to verify it's purity by the determination of the ratio absorbance at 260/280nm.

2.2 Salmon Sperm DNA solution

Salmon sperm DNA solution was prepared in PBS buffer (10mM, pH 7.4) to a final concentration of 5×10^{-4} M from a stock solution (10mg/mL) of UltraPure™ Salmon Sperm DNA Solution (Invitrogen).

3. Preparation of lipoplexes

The DNA concentration used for cell transfection and lipoplex cytotoxicity assays was 1ug/ul and for the complexation and stability studies was 20ug/ul. The balance between charges is given by the charge ratio (+/-):

$$C.R.(+/-) = \frac{[+]}{[-]} = \frac{[Ammonium\ groups\ of\ DODAX + Amine\ groups\ of\ DC - Chol]}{[Phosphate\ groups\ from\ DNA]}$$

The positive charges are given by the concentration of ammonium groups present in DODAB/C and amine present in DC-Chol. The negative charges are given by the concentration of phosphate groups in DNA, which correspond to the nucleotide concentration.

For the mean size measurements, DODAC:MO:DC-Chol (ratios 6:3:1, 5:4:1, 4:1:1) and DODAB/C:MO (ratio 2:1) were added in a single step, at 25ºC, to sperm salmon DNA (20ug/ul), forming lipoplexes with charge ratios (+/-) of 0.5, 1.0, 1.5, 2.0, 3.0, 4.0. The addition was followed by a 5 minutes agitation period. Size measurements and Zeta Potential determination of the lipoplexes were performed after 1h of incubation.

For the FRET assay, liposomes were incubated with rhodamine DHPE (5×10^{-5} M) at a 1:200 ratio during their preparation by the film hydration method followed by esxtrusion, as previously described. The Salmon sperm DNA (20µg/µL) was also incubated with BOBO-1 probe (2×10^{-5} M). The lipoplex preparation method and the liposome formulations used were the same as the ones produced for the complexation studies forming lipoplexes with charge ratios (+/-) of 0.25, 0.5, 0.75, 1.0, 1.5, 2.0, 2.5, 3.0 and 4.0.

For stability studies, the lipoplex formation procedure and the used liposome formulations were the same as described before, except for the generated charge ratios (+/-), which was 4.0.

The liposomes used to form the DNA-liposome complexes for the complexation assays, FRET assay and stability studies were produced only by the film hydration method followed by extrusion.

For the transfection and cytotoxicity assays, lipoplexes were generated at a charge ratio (+/-) of 4.0 and plasmid DNA (1µg/µL) was used. The liposomes used in the production of these complexes were produced by the ethanolic injection and the film hydration method described previously and the used formulations were DODAB/C:MO:DC-Chol (ratios 6:3:1, 5:4:1, 4:1:1) and DODAB/C:MO (ratio 2:1).

4. Dynamic Light Scattering and Zeta potential measurements

4.1 Dynamic Light Scattering (DLS) assays

Dynamic Light Scatering (or PCS - Photon Correlation Spectroscopy) is a technique that measures the Brownian motion of the particles by illuminating them with a laser and analyzing the intensity fluctuations in the scattered light, relating these measurements to the size of the particles. It can also give us information about the homogeneity of the sample (Malvern, 2005). Particles suffer random movement in a liquid due to the surrounding molecules which bombard them and this random movement is defined as the Brownian motion. The speed of movement is used to determine the size of the particle. A laser beam is used to irradiate the randomly moving particles in order to record the intensity of the light scattered in a fixed or variable angle and in given time interval. Smaller particles move faster, so they lead to faster intensity fluctuations due to their high diffusion coefficient. Larger particles move slower, is manifested in slower intensity fluctuations. (Clark *et al*, 1970; Malvern, 2005).

The Stokes-Einstein equation defines the relationship between the particle size and its speed due to the Brownian motion:

$$D = \frac{\kappa \mathrm{T}}{6\pi R\eta}$$

Where (D) is the particle diffusion coefficient, (K) the Boltzmann constant, (T) the temperature, (R) the radius of the particle and (η) the medium viscosity

The Polydispersity index (PDI) is an indicative of the heterogeneity degree regarding to the sample size. The PDI values ranges between 0.0 and 1.0 and depend on the range of diameters found between the average diameter. The greater this range of diameters is, the higher the standard deviation, thus, the greater is the PDI (Malvern, 2005).

Measurements of particle size and PDI were performed using a Malvern zetasizer Nano SZ particle analyzer (Malvern Instruments).

4.2 Mean size of liposomes

1 mL solutions of DODAB/C:MO (2:1), DODAC:MO:DC-Chol (DODAC:MO (2:1) with 10%, 20%, 30%, 40% and 50% of DC-Chol) and DODAB/C:MO:DC-Chol (ratios 6:3:1, 5:4:1, 4:1:1) liposomes, produced by ethanolic injection and the film hydration method followed by extrution, were placed in disposable polystyrene cuvettes (Sarstedt, Germany) for DLS measurements. These measurements were performed at 25ºC in a Malver ZetaSizer Nano SZ particle analyser. The Malvern Dispersion Technology Software (DTS) was used and mean size (nm) average, polydispersity index and error values were taken in consideration.

4.3 Mean size of lipoplexes

1 mL solutions with DNA/DODAB/C:MO (2:1) and DNA/DODAC:MO:DC-Chol (ratios 6:3:1, 5:4:1, 4:1:1) lipoplexes at charge ratios (+/-) 1.0,1.5, 2.0,3.0 and 4.0 were placed in disposable polystyrene cuvettes (Sarstedt, Germany) for DLS measurements, which were executed in a Malver ZetaSizer Nano SZ particle analyser, at 25ºC. The mean size (nm) average, polydispersity index and error values were taken in consideration and data was analyzed with Malvern Dispersion Technology Software (DTS).

4.4 Stability studies

4.4.1 Stability in serum

Solutions of DODAC:MO:DC-Chol (ratios 6:3:1, 5:4:1, 4:1:1) and DODAB/C:MO (2:1) liposomes, prepared by the film hydration method, described previously, were incubated in 0, 30 and 80% of FBS (Fetal Bovine Serum) and were placed in disposable polystyrene cuvettes for DLS measurements. The same procedure was repeated for DNA/DODAC:MO:DC-Chol (ratios 6:3:1, 5:4:1, 4:1:1) and DNA/DODA(X):MO (2:1) lipoplex solutions at charge ratio (+/-) 4.0. The measurements were executed at different time points: 0h, 3h, 6h and 24h. The cuvettes were placed in a Malver ZetaSizer Nano SZ particle analyser and again, the Malvern Dispersion Technology Software (DTS) was used and mean size (nm) average, polydispersity index and error values were taken in consideration.

4.4.2 Stability in NaCl

Solutions of DODAC:MO:DC-Chol (ratios 6:3:1, 5:4:1, 4:1:1) and DODAB/C:MO (2:1) liposomes, prepared by the film hydration method followed by extrusion, as described above, were incubated with different volumes of a NaCl solution (3.84M) in order to obtain different final concentrations of this salt (0, 5, 10, 50, 100 and 150 mM). The same procedure was applied to the lipoplex solutions. The DNA/DODAC:MO:DC-Chol (ratios 6:3:1, 5:4:1, 4:1:1)/DNA complex and DNA/DODAB/C:MO (2:1)/DNA complex solutions at charge ratio (+/-) 4.0, were incubated with the same NaCl solution as the liposomes in order to obtain lipoplex solutions incubated with different concentrations of NaCl (0, 5, 10, 50, 100 and 150 mM). The samples were placed in disposable polystyrene cuvettes for DLS measurements which took place 1h after incubation. The cuvettes were placed in a Malver ZetaSizer Nano SZ particle analyser and again, the Malvern Dispersion Technology Software (DTS) was used and mean size (nm) average, polydispersity index and error values were taken in consideration.

5. Zeta (ζ) Potential assays

The zeta potential is a physical property found in particles in suspension. When in contact with a liquid, particles tend to acquire an electrical charge on their surface. There is an electrical double layer surrounding each particle in suspension. There are two parts of this layer: the stern layer, where the ions are strongly bound, and the diffuse layer where the ions are not so firmly attached (Figure 10). In this last layer, there is a boundary where the ions and particles form a stable entity. When the particle moves, only the ions within this boundary, the slipping plane, move with it. The potential in the slipping plane is known as the zeta potential (Malvern, 2005).

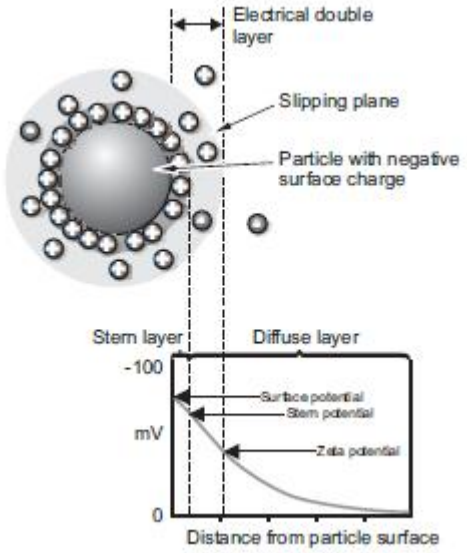


Figure 10. Schematic representation of the double layer surrounding a particle in suspension (Malvern, 2005).

The zeta potential is not obtained by a direct measurement. It is calculated by the determination of the electrophoretic mobility, obtained by performing electrophoresis experiments on the samples and executing measurements of the particles velocity using Laser Dopller Velocimetry (LDV), and then applying the Henry equation:

$$\mu e = \frac{2\varepsilon\zeta - potencialf\ (Ka)}{3\eta}$$

where, μe is the electrophoretic mobility, the zeta (ζ) potential, ε is the dielectric constant, f (Ka) is the role of Henry and η is the viscosity of the medium (Malvern, 2005).

The electrophoretic mobility is measured by applying an electric field. The potential difference created by the opposite charges in the two electrodes makes the charged particles that exist in suspension move to the electrode of opposite charge. The particles will move with a constant velocity when equilibrium between these two forces is reached. The velocity at which the particles move in an electric field is known as electrophoretic mobility. This velocity is measured by Laser Dopller Velocimetry (LDV) (Malvern, 2005).

5.1 Zeta potential of the liposomes

1 mL solutions of DODAB/C:MO (2:1) and DODA(X):MO:DC-Chol (ratios 6:3:1, 5:4:1, 4:1:1) liposomes were placed in 0.7ml universal dip cells for ζ-potential measurements, which took place at 25ºC in a Malver ZetaSizer Nano SZ particle analyzer. The Malvern Dispersion Technology Software (DTS) was run with monomodal mode data processing and ζ-potential (mV) average and error values were taken into consideration.

5.2 Zeta potential of lipolplexes

1 mL solutions of DNA/DODAB/C:MO (2:1) and DNA/DODAB/C:MO:DC-Chol (ratios 6:3:1, 5:4:1, 4:1:1) lipoplexes at charge ratios (+/-) 0.5, 1.0, 1.5, 2.0, 3.0 and 4.0 were placed in 0.7ml universal dip cells (Malvern Instruments) for ζ-potential measurements, which were executed in a Malver ZetaSizer Nano SZ particle analyzer, at 25ºC. The Malvern Dispersion Technology Software (DTS) was run with monomodal mode data processing and ζ-potential (mV) average and error values considered.

5.3 Stability studies

5.3.1 Stability in NACl

Solutions of DODAC:MO:DC-Chol (ratios 6:3:1, 5:4:1, 4:1:1) and DODAB/C:MO (2:1) liposomes, prepared by the film hydration method followed by extrusion as described above, were incubated with different volumes of a NaCl solution (3.84M) in order to obtain different final concentrations: 0, 5, 10, 50, 100 and 150 mM. For DNA/DODAC:MO:DC-Chol (ratios 6:3:1, 5:4:1, 4:1:1) and DNA/DODAB/C:MO (2:1) lipoplex solutions at charge ratio (+/-)4.0, it was applied the procedure previously described for the liposome solutions. The samples were placed in 0.7ml universal dip cells (Malvern Instruments) for ζ-potential measurements which took place 1h after incubation. The cells were placed in a Malver ZetaSizer Nano SZ particle analyser and again, the Malvern Dispersion Technology Software (DTS) was used and and ζ-potential (mV) average and error values were taken in consideration

6. Fluorescence Resonance Energy Transfer (FRET) assay

FRET is a technique that can describe energy transfer between two chromophores. There is an energy transfer between a donor chromophore, initially in its electronic exited state, and an acceptor chromophore. The amount of energy transfer is dependent on the distance between the two chromophores.Also the absorption spectrum of the acceptor must overlap with the emission spectrum of the donor in order for the non-radiative transfer of excitation energy occurs (Figure 11). This way, several vibronic transitions in the donor and the acceptor have practically the same energy (Valeur, 2001). Other criterion that must be satisfied for FRET to occur are that the donor and the acceptor must have approximately parallel transition dipole orientations and that the donors fluorescence lifetime must have a sufficient duration in order to allow the FRET to occur (Piston *et al.*, 1948; Valeur, 2001).

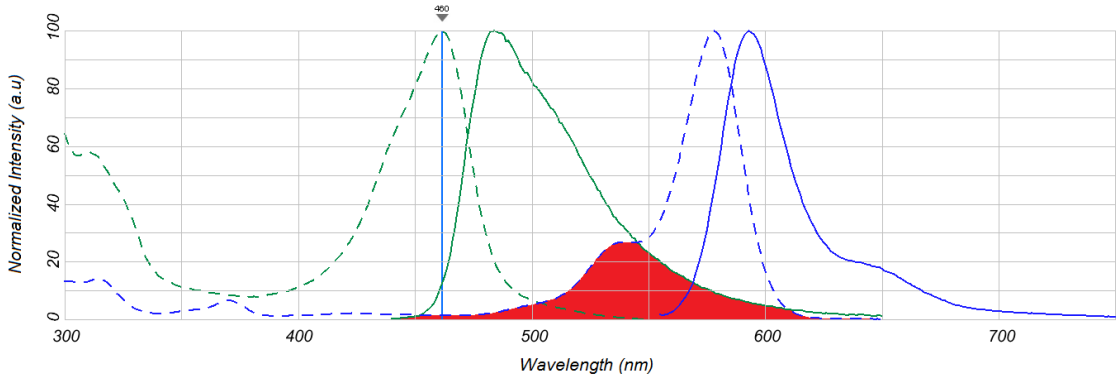


Figure 11. Donor (BOBO-1) and acceptor (rhodamine-DHPE) spectrums with the spectral overlap integral between the emission spectrum of the donor and the absorption of the acceptor. (adapted from: http://www.invitrogen.com/site/us/en/home/support/ResearchTools/Fluorescence-SpectraViewer.html

FRET is widely used to measure distances between donors and acceptors in macromolecular systems. The efficiency process depends on the inverse sixth distance between the donor and acceptor.

$$E = Ro^6/(Ro^6 + r^6)$$

Where Ro is the critical radius of Förster, which can be defined as the distance at which half the energy is transferred and r is the actual distance between donor and acceptor. So, it's easy to see that the distance affects greatly the efficiency of energy transfer. The critical radius of Förster (R_0) can be estimated by:

$$R_0\ (nm) = 979\ (\kappa^2\ \eta^4\ \phi_0\ \zeta)^{1/6}$$

Where η is the refractive index of the medium, ϕ_0 is the fluorescence quantum yield of the donor, ζ the spectral overlap integral and κ an orientation factor.

The energy transfer rate (Φ_{FRET}) can be determined by:

$$\Phi_{FRET} = 1/\tau_d\ (R_0/r)^6$$

Where τ_d is the decay time of the donor fluorescence in the absence of an acceptor and r is the distance between donor and acceptor.

This assay was executed by monitoring the decrease of fluorescence of BOBO-1 (donor) in the presence of rhodamine DHPE (acceptor) in a Horiba Jobin Yvon Spex Fluorolog spectrofluorimeter after the addition of the cationic liposomes to the DNA solutions, followed by a 5 minutes agitation period with a magnetic stirrer. The fluorescence intensities were determined at λ_{exc} = 460 nm with spectral bandwidths of 1 nm. All emission spectra were integrated, and the ratio of the areas for the dye solutions were determined. The fluorescence resonance energy transfer efficiency (ΦFRET) was determined from the following expression:

$$(\Phi FRET) = (1 - F_{(DA)} / F_{(D)}) * 100$$

Where $F_{(DA)}$ and $F_{(D)}$ are the fluorescence emission of donor in the presence and absence of acceptor, respectively.

7. Cell Culture

The 293 T and L929 cell lines were cultured in DMEM medium supplemented with 10% (v/v) heat-inactivated FBS and 1% (v/v) of an antibiotic/antimycotic solution, with 5 % CO2 at 37 ºC.

7.1 MTT assay

This technique involves the use of MTT (3,(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide)) which is a tetrazolium salt. This salt is metabolized by mitochondrial enzyme activity in living cells, forming an insoluble coloured formazan salt, thus being a rapid colorimetric method to assess cell viability (Masters, 2000).

Briefly, after an incubation period of 48h, the culture medium was replaced by fresh medium to which it was added 50µL of MTT 10x to each well. Cell cultures were then incubated for 2h in a humidified incubator at 37ºC, with 5% CO2. Then, the culture medium containing MTT was removed and it was added to each well 500µL of a DMSO/ethanol solution (1:1(v/v)), followed by a slight agitation of the plate in order to facilitate the dissolution of the crystals. It was taken from each well 150µL of the solution containing the dissolved crystals and it was placed in a 96 well reading plate in

order to read the optical density at 570nm in a microplate reader SpectraMax Plus (Molecular Devices, USA). The DMSO/ethanol solution (1:1(v/v)) was used as blank.

7.2 LDH (lactate dehydrogenase) assay

This technique allows us to make an indirect measurement of cell viability. LDH is a quantitative measurement of cell viability loss since it is released by dead or dying cells. LDH measurement basically follows the oxidation of NADH, which is initiated by the addition of pyruvate, by the change of absorbance at 340nm. These measurements are performed in a cell-free medium at a temperature of 30ºC (Masters, 2000).

So, after a 48h incubation period, the cell culture medium from each well was collected to individual eppendorfs in order to determine extracellular LDH. The eppendorfs were then centrifuged at 13000rpm for 1 minute in order avoid having cells or cellular fragments in suspension and the supernatant was collected to fresh eppendorfs. Then, 40 µl of supernatant were placed in a 96 well reading plate, along with 250ul of NADH solution (0.3mM in phosphate buffer 0.05M, pH 7.4) and 10 µl of pyruvate solution (0.05M in phosphate buffer 0.05M, pH 7.4) in each well. The pyruvate solution was added immediately before performing the optical density reading in a multiwall plate reader. The readings were executed at 30ºC in intervals of 10 seconds for 3 minutes at 340nm in a microplate reader SpectraMax Plus (Molecular Devices, USA).

7.3 Cytotoxicity assay

Cytotoxicity was assessed by MTT assay and by the measurement of extracellular Lactate Dehydrogenase (LDH), after a 48h hour period of incubation with the liposomes and lipoplexes. The 293T cell line was used to evaluate the cytotoxicity of the lipoplexes and the L929 cell line was used to evaluate liposome cytotoxicity.

Cells were cultured in 24-well culture plates (TPP, Trasadingen, Switzerland) at a density of 3×10^4 cells per well and 1×10^5 cells per well for liposomes and lipoplexes respectively. The cell culture medium was removed and replaced with fresh medium immediately before the liposomes and lipoplexes addition. To the plaque with the L929 cell line, it was added to each well the liposome solution of interest (buffered

with PBS 1x, pH 7.4) with different concentrations. For the DODAB/C:MO:DC-Chol (ratios 6:3:1, 5:4:1, 4:1:1) and DODAB/C:MO (ratio 2:1) the final concentrations were 5, 50 and 100 ug/ml. Cells incubated with DMSO (30% of the total volume), a volume of PBS solution (1x, pH 7.4) corresponding to the volume added for the maximum concentration of liposomes for each formulation and cells without the addition of any compounds were used as controls. To the plaque with 293T cell line, 100 µL of lipoplex solution prepared in Opti-MEM (Gibco) was added to each well, in duplicate. The lipoplexes used were obtained from the addition to DNA of the same liposome formulations used for the transfection experiments, with a charge ratio (+/-) of 4.0. Cells incubated with DMSO (30% of the total volume) and cells without the addition of the compounds were used as controls. Cell cultures were then maintained in a humidified incubator, with 5% CO2, at 37ºC for 48h. After that period of incubation, the extracellular LDH and the MTT assays were performed in a microplate reader SpectraMax Plus (Molecular Devices, USA).

7.4 Transfection

293T cells were seeded into 24-well plates (TPP, Trasadingen, Switzerland), at a density of 1×10^5 cells per well. Just before adding to each well 100µl of the lipoplex solutions prepared in Opti-MEM (Gibco), the cell culture medium was replaced by fresh medium. The cells cultures were kept in a humidified incubator with 5% CO2, at 37ºC. EzWay transfection agent (Koma Biotech) was also used to carry out DNA transfection, and used according to the manufacturer's instructions. After 48h, the β-galactosidase expression was evaluated with the β-Galactosidase Enzyme Assay System with Reporter Lyses Buffer (Promega), according to the standard given protocol. The amount of protein in each sample was also quantified by the Bradford method to normalize for varying cell numbers resulting, for example, of cytotoxicity.

7.5 Bradford protein quantification assay

This is a method that allows protein determination by the binding of Coomassie Brilliant Blue G-250 to the proteins. The dye has two forms: blue and red form. When the dye binds to a protein, it will cause a shift of the absorption maximum of the dye

from 465nm to 565 nm, and the red form of the dye is converted in the blue form. An excess amount of dye is added to the protein samples and the absorption at 595nm is measured. As control, protein standards are used, enabling the determination of the amount of protein in unknown samples (Bradford, 1976).

Briefly, a standard curve was made by preparing samples of BSA with final concentrations of 0, 0.25, 0.5, 0.5, 1 and 2 mg/mL and 10µL of each of these samples with different concentrations were added to individual wells in a 96 well reading plate. The same procedure was applied to the samples with unknown amount of proteins. Then, 250uL of Bradford reagent was added to each well and the contents mixed by vortexing. The absorbance at 595nm was measured after 5 minutes in a microplate reader SpectraMax Plus (Molecular Devices, USA) against a reagent blank prepared from the Reporter Lyses Buffer (RLB) solution (Promega).

III. Results and Discussion

1. Dynamic Light Scattering (DLS) assays

One of the main of this work was to study how the counter ion exchange (Bromide or Chloride), the preparation method (ethanolic injection and film hydration followed by extrusion) and also the inclusion of DC-Cholesterol affects the mean size and charge of the DODAB/C:MO based liposomes and (DODAB/C:MO:pDNA) lipoplexes.

1.1 Mean size of liposomes

As reported in previous work by Neves Silva, lipoplexes based DODAB:MO (2:1) liposomes prepared by ethanolic injection have shown good transfection efficiency (Neves Silva *et al*., 2011) the DODAB:MO (2:1) formulation was chosen to be used as means of comparison in this work.

The figure 12 presents the mean size and PDI for DODAB:MO (2:1) and DODAC:MO (2:1) liposomes prepared by two preparation methods.

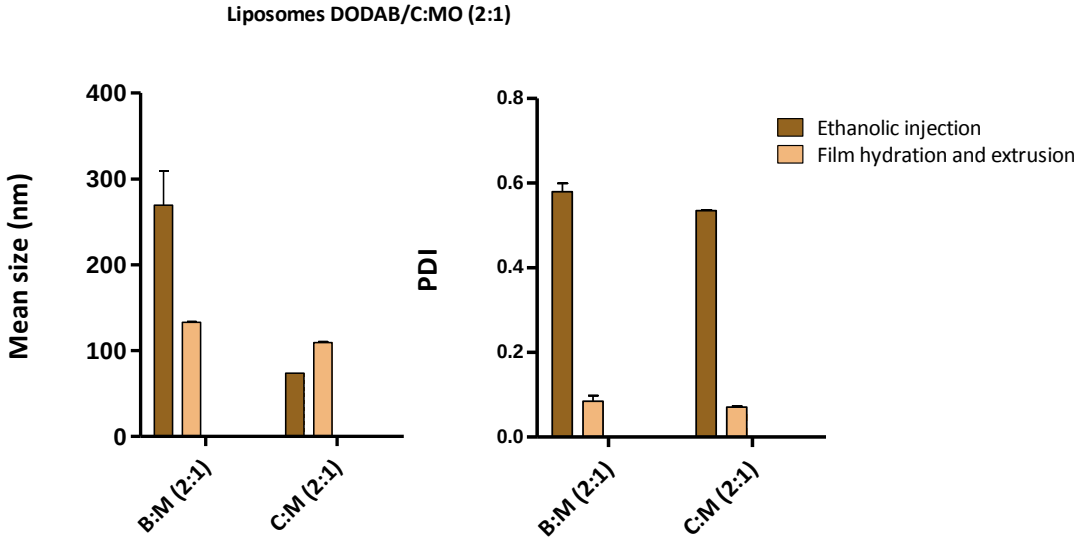


Figure 12. Mean size and Polidispersity index (PDI) of DODAB/C:MO (2:1) liposomes produced by ethanolic injection and Film hidration method followed by extrusion. B:M (2:1) – DODAB:MO liposomes with 2:1 ratio; C:M (2:1) – DODAC:MO liposomes with 2:1 ratio.

It seems that the liposomes prepared by the extrusion method, show no significant differences in the mean size or in the PDI of the populations of DODAB:MO and DODAC:MO liposomes, although DODAB:MO liposomes present slightly larger mean size and PDI values (132.8 nm and 0.085 respectively) compared with the DODAC:MO vesicles (109.5 nm and 0.071 respectively).

Regarding to liposomes produced by ethanolic injection, DODAB:MO liposomes, seem to present a higher mean size (~270nm) compared with DODAC:MO (~74nm). although both formulation present high PDI values

From figure 12 it can be observed that DODAB:MO liposomes prepared by ethanolic injection present higher mean size than the DODAB:MO liposomes prepared by extrusion, however, for DODAC:MO liposomes the opposite effect is observed. These changes could be related with the type of liposomes prepared by the two methods. It is important to note that the liposomes produced by ethanolic injection are multivesicular liposomes as opposed to the ones produced by film hydration and submitted to extrusion, which are unilamellar.

The measurements performed with extruded liposomes indicated the presence of only one population, as it was to be expected, hence the low PDI values but for liposomes produced by ethanolic injection three different populations were observed (see Appendix 1). It should be noticed that the main population found in DODAB:MO liposomes produced by ethanolic injection was about 246nm, still higher than the main population found in DODAC:MO lipossomes produced by the same method, which was around 181 nm (see Appendix 1). But the value of the main population of DODAC:MO liposomes produced by ethanolic injection is higher than the value registered for the mean size using DODAC:MO liposomes produced by the extrusion method

It seems that the exchange of the counter ion Bromide with Chloride influences the size of the liposomes produced by both methods, making them smaller.

These results come in line with results obtained previously on the investigation of extruded vesicles of DODAB and DODAC where the authors explain that this difference in vesicle size might be explained by the specificity of the counterion binding to the vesicle interfaces (Lopes *et al*., 2008). This data is also in agreement with reports for spontaneous, injected and sonicated dispersions. The specificity of counter ion binding is also reflected in T_m . Bromide binds DODAB vesicles yelding larger

vesicles with lower curvature and T_m than DODAC vesicles. This means that DODAB vesicles present a more densely packed bilayer, which allows Bromide to bind more tightly to the vesicle interfaces than Chloride (Feitosa *et al.*, 2000)

1.1.1 Effect of DC-Cholesterol

In light of these results, it was decided to study the effect of the inclusion of DC-Chol on the mean size and PDI of DODAC:MO (2:1) liposomes, prepared by extrusion method, with 10%, 20%, 30%, 40% and 50% of DC-Chol.These results are represented in figure 13.

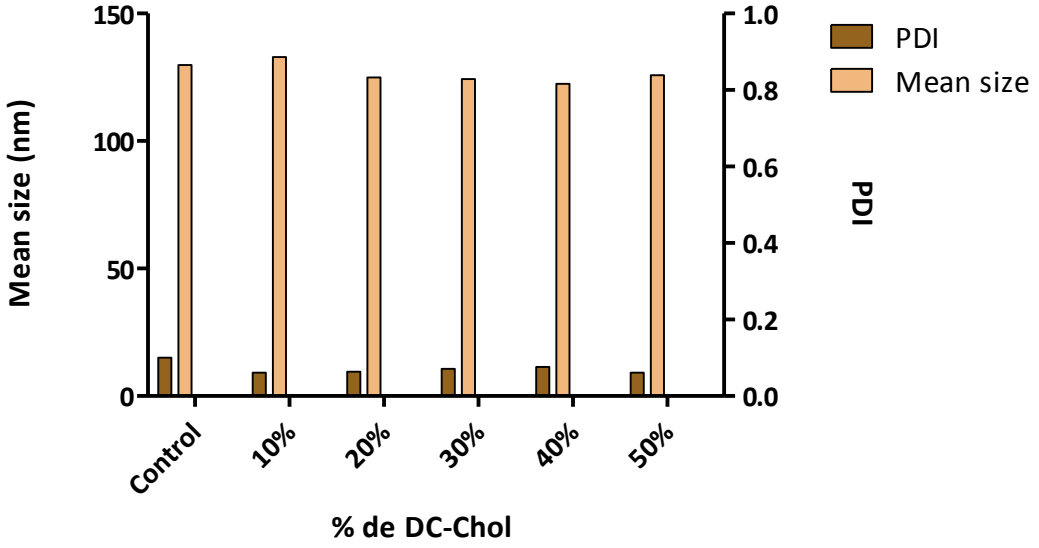


Figure 13. Mean size and polidispersity index of DODAC:MO (2:1) extruded liposomes with diferente percentages of DC-Chol (B). Control: DODAC:MO (2:1) liposomes.

According to these results, the inclusion of different percentages of DC-Chol in the DODAC:MO system doesn't alter significantly the size or the PDI of extruded vesicles.

Since the inclusion of DC-Chol in the formulation has the purpose molding the membranes fluidity and eventually aid in an enhancement of transfection efficiency, it was decided not to include high percentages of DC-Chol in the system, since it has

been shown before that high levels of cholesterol in the liposomal formulation lead to the formation of rigid domains (Hungerford *et al*., 2005), altering the systems fluidity.

Taking this in mind it was decided to include 10% and 16% of DC-Chol in the studied formulations. So, the formulations DODAB/C:MO:DC-Chol in the molar ratios of 6:3:1, 5:4:1, 4:1:1 will be studied.

The Figure 14 presents the mean size and PDI of liposomes composed by DODAB/C:MO:DC-Chol in the molar ratios of 6:3:1, 5:4:1, 4:1:1, produced by the ethanolic injection method and film hydration method followed by extrusion compared with the DODAB/C:MO (2:1).

The inclusion of cholesterol in the DODAC:MO formulation prepared by the two preparation methods (ethanolic vs extrusion) does not promotes a great change in the mean size of the liposomes. Again, the liposomes prepared by the ethanolic injection presenting a very high PDI. The formulation 6:3:1 was the one that present a higher mean size.

Moreover, it is important to refer that the readings performed to ethanolic injection liposomes showed the presence of more than one population, thus explaining the high values of PDI (see Appendix 1). Also important is that this mean size represent a virtual value, since there are populations of various sizes found in solution for liposomes produced by ethanolic injection.

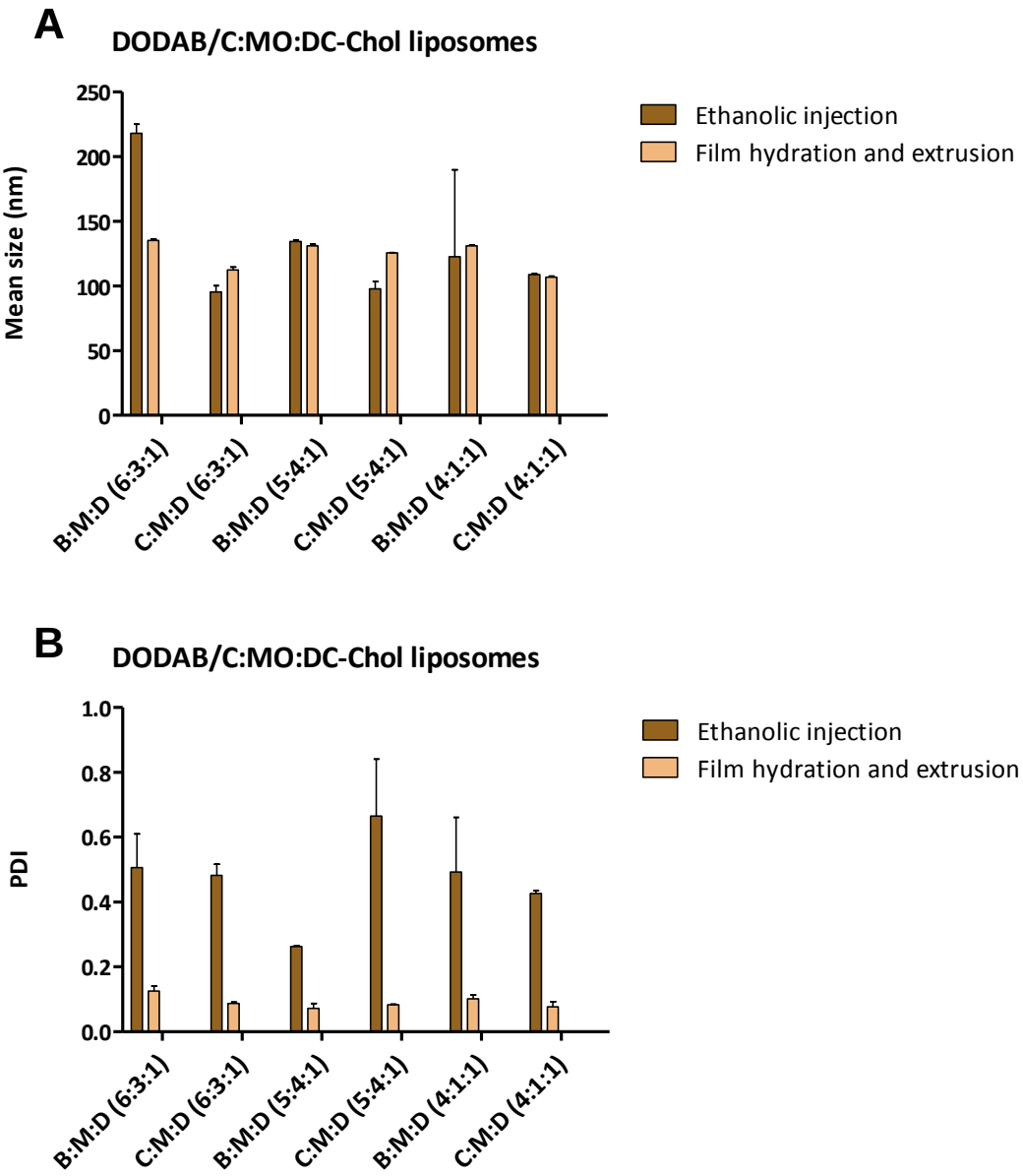


Figure 14. Mean size (A) polidispersity index (B) of DODAB/C:MO (2:1) and DODAB/C:MO:DC-CHOL (6:3:1, 5:4:1 and 4:1:1) liposomes produced by ethanolic injection and by film hydration followed by extrusion. B:M:D (6:3:1) – DODAB:MO:DC-Chol liposomes with 6:3:1 ratio; C:M:D (6:3:1) – DODAC:MO:DC-Chol liposomes with 6:3:1 ratio; B:M:D (5:4:1) – DODAB:MO:DC-Chol liposomes with 5:4:1 ratio; C:M:D (5:4:1) – DODAC:MO:DC-Chol liposomes with 5:4:1 ratio; B:M:D (4:1:1) – DODAB:MO:DC-Chol liposomes with 4:1:1 ratio; C:M:D (4:1:1) – DODAC:MO:DC-Chol liposomes with 4:1:1 ratio.

1.1.2 Stability in time of liposomes and lipoplexes

In order to know how the preparation methods affect the liposomes stability with the course of time, measurements of particle size were performed throughout time.

As it can be observed in figure 15, the liposomes produced by film hydration followed by extrusion are very stable, maintaining a constant size along time. The PDI also doesn't suffer changes through time (see Appendix 2).

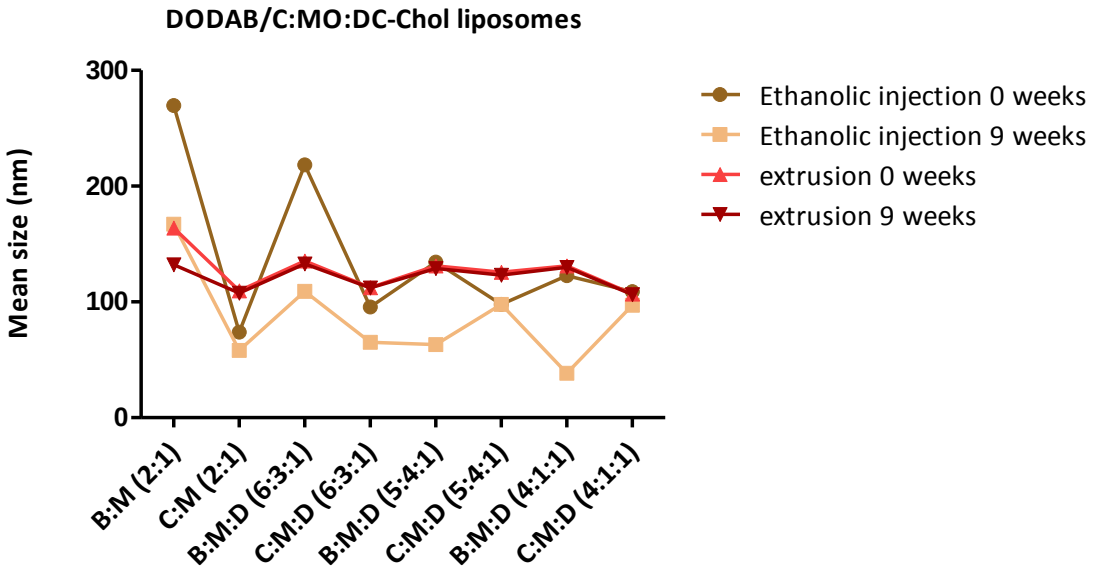


Figure 15. Comparison of the mean size of DODAB/C:MO (2:1) and DODAB/C:MO:DC-CHOL (6:3:1, 5:4:1 and 4:1:1) liposomes produced by ethanolic injection and by film hydration followed by extrusion over 9 weeks. B:M (2:1) – DODAB:MO liposomes with 2:1 ratio; C:M (2:1) – DODAC:MO liposomes with 2:1 ratio; B:M:D (6:3:1) – DODAB:MO:DC-Chol liposomes with 6:3:1 ratio; C:M:D (6:3:1) – DODAC:MO:DC-Chol liposomes with 6:3:1 ratio; B:M:D (5:4:1) – DODAB:MO:DC-Chol liposomes with 5:4:1 ratio; C:M:D (5:4:1) – DODAC:MO:DC-Chol liposomes with 5:4:1 ratio; B:M:D (4:1:1) – DODAB:MO:DC-Chol liposomes with 4:1:1 ratio; C:M:D (4:1:1) – DODAC:MO:DC-Chol liposomes with 4:1:1 ratio.

The ethanolic injection liposomes are not stable during long periods of time though. In fact, the mean size of the mean size decreased in all formulations but the PDI value increased (see Appendix 2). This behavior may be possibly due to the fusion of the multivesicular vesicles.

1.2 Mean size of lipoplexes

Figures 16 and 17 present the mean size and polidispersity index (PDI) of lipoplexes prepared using salmon sperm DNA and DODAB/C:MO (2:1) and DODAC:MO:DC-Chol (6:3:1, 5:4:1, 4:1:1) liposomes produced by the extrusion method.

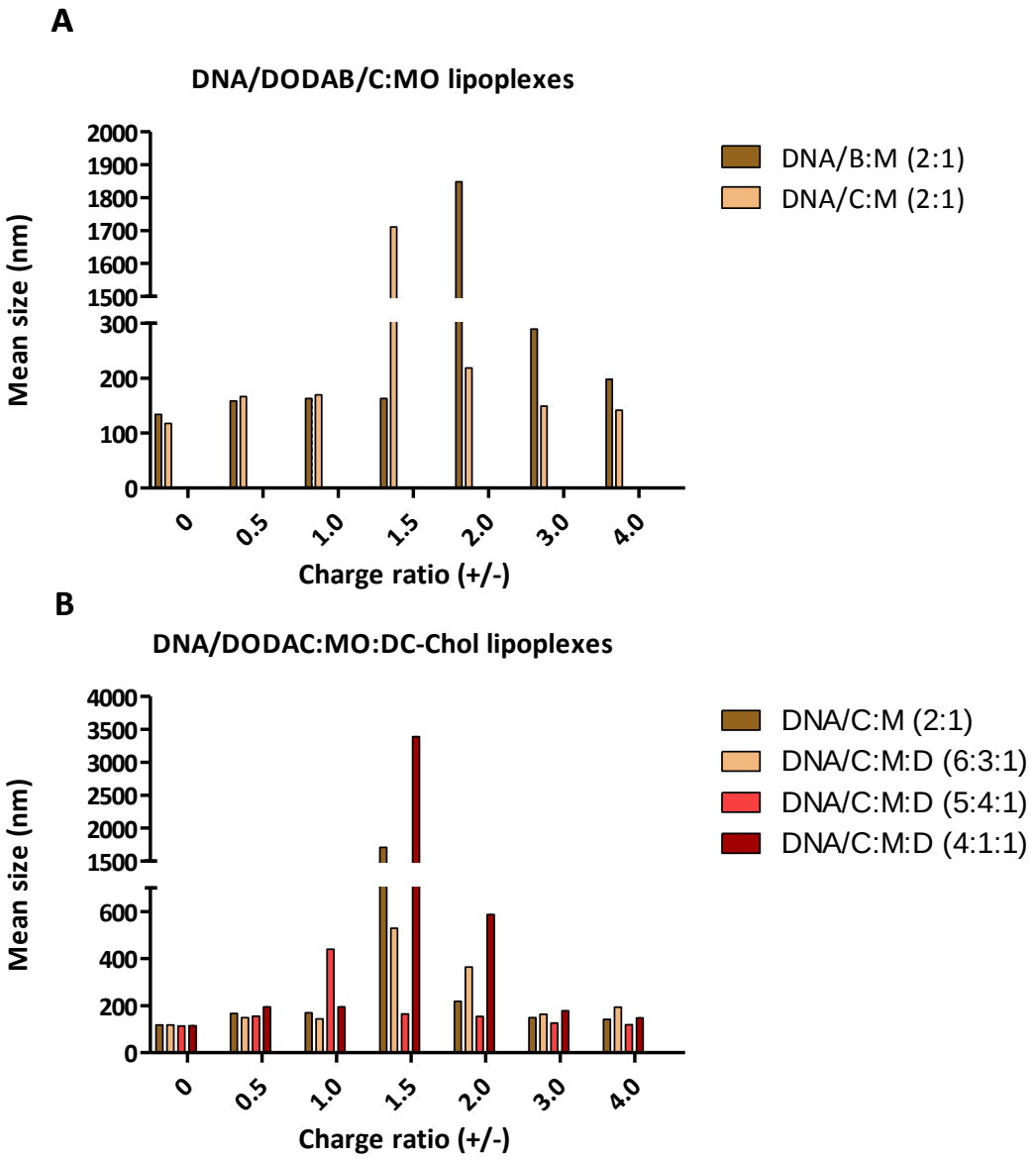


Figure 16. Mean size of :(A) DNA/DODAB/C:MO (2:1) lipoplexes; (B) DNA/DODAC:MO (2:1) and DNA/DODAC:MO:DC-Chol (6:3:1, 5:4:1 and 4:1:1) lipoplexes at different charge ratios (+/-). DNA/B:M (2:1) – DNA/DODAB:MO (2:1) lipoplexes; DNA/C:M (2:1) – DODAC:MO (2:1) lipoplexes; DNA/C:M:D (6:3:1) – DNA/DODAC:MO:DC-Chol (6:3:1) lipoplexes; DNA/C:M:D (5:4:1) – DNA/DODAC:MO:DC-Chol (5:4:1) lipoplexes; DNA/C:M:D (4:1:1) – DNA/DODAC:MO:DC-Chol (4:1:1) lipoplexes.

The PDI values also seem to follow the tendency observed for the mean size values (Figure 17).

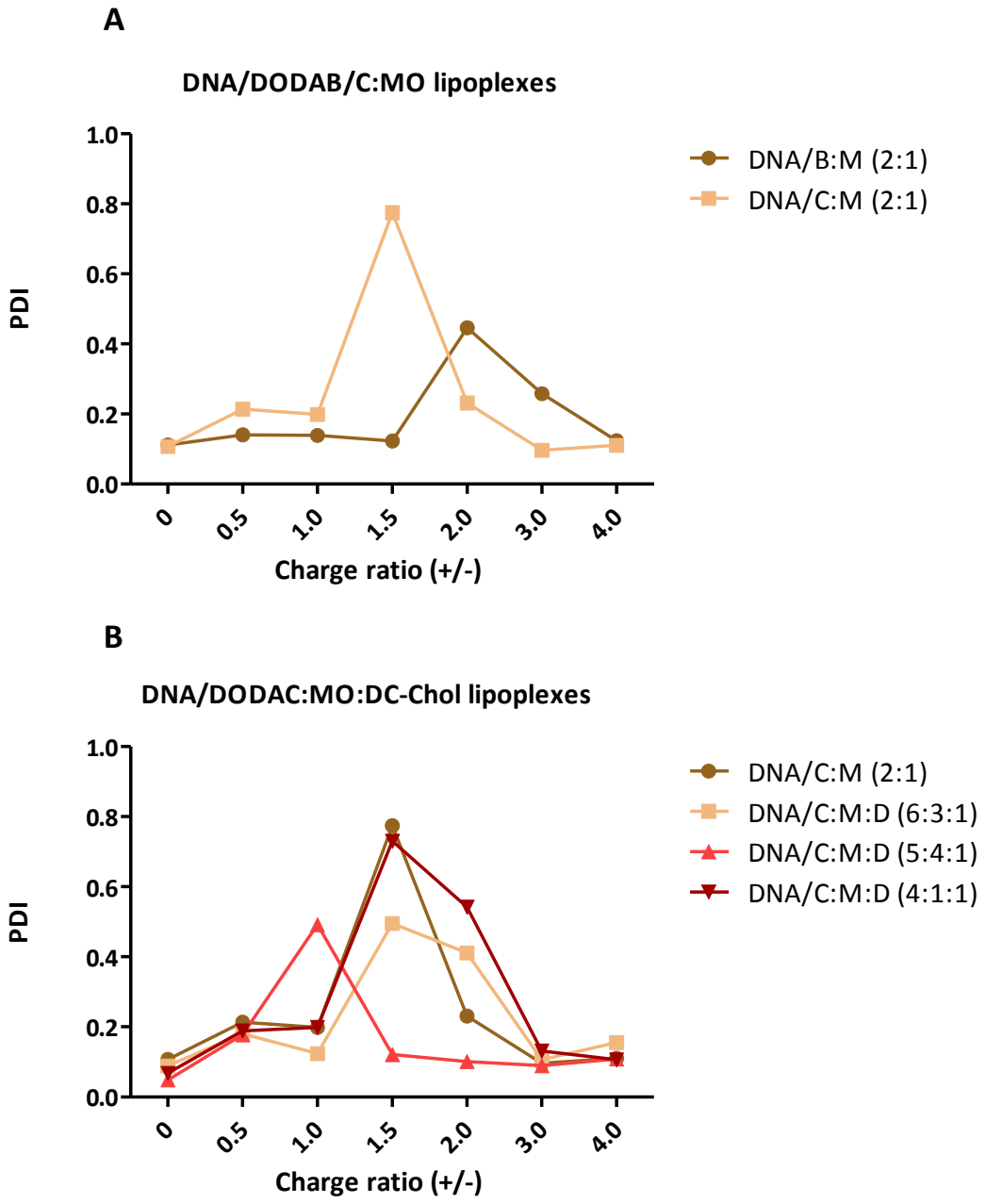


Figure 17. Polidispersity index of :(A) DNA/DODAB/C:MO (2:1) lipoplexes; (B) DNA/DODAC:MO (2:1) and DNA/DODAC:MO:DC-Chol (6:3:1, 5:4:1 and 4:1:1) lipoplexes at different charge ratios (+/-). DNA/B:M (2:1) – DNA/DODAB:MO (2:1) lipoplexes; DNA/C:M (2:1) – DODAC:MO (2:1) lipoplexes; DNA/C:M:D (6:3:1) – DNA/DODAC:MO:DC-Chol (6:3:1) lipoplexes; DNA/C:M:D (5:4:1) – DNA/DODAC:MO:DC-Chol (5:4:1) lipoplexes; DNA/C:M:D (4:1:1) – DNA/DODAC:MO:DC-Chol (4:1:1) lipoplexes.

It can be observed a great increase in mean size with the increase in CR (+/-) followed by a decrease in mean size. This increase in the mean size is different for each formulation.

DNA/DODAC:MO (2:1) reaches the higher size at the charge ratio (+/-) 1.5 compared with pDNA/DODAB:MO (2:1) and this occurs at the charge ratio (+/-) 2.0. Again the lipoplexes obtained from DODAC:MO (2:1) liposomes are smaller than lipoplexes obtained from DODAB:MO (2:1) liposomes at the charge ratios (+/-) 3.0 and 4.0 .

For DNA/DODAC:MO:DC-Chol (5:4:1) complexes the increase in size occurs at the charge ratio (+/) 1.0 compared with pDNA/DODAC:MO:DC-Chol (6:3:1) and (4:1:1) complexes that occurs at charge ratio (+/-) 1.5. The mean size of the lipoplexes at the charge ratios (+/-) 3.0 and 4.0 are similar for all the formulations. (Figure 16).

The increase in size at a specific charge ratio (+/-) is associated with the point at which is reached an isoelectric point.

At charge ratios (+/-) 3.0 and 4.0, the lipoplexes mean size and PDI stabilize, probably because at these two charge ratios (+/-), most of the DNA is complexed.

Comparing the DNA/DODAB:MO (2:1) and DNA/DODAC:MO (2:1) lipolplexes , it seems that the exchange of bromide to chloride influences isoeletric point. In DNA/DODAC:MO (2:1) complexes, this increase occurs at a lower charge ratio (+/-). Also, at charge ratios (+/-) 3.0 and 4.0, the DNA/DODAC:MO (2:1) lipoplexes appear to be smaller, so the exchange of Bromide to Chloride seems to lead to the formation of smaller complexes. This behavior may be associated either the size/charge or fluidty of the liposomes.

The inclusion of DC-Chol in the liposomal formulation does not appear to exert great changes in terms of lipoplex mean size. When comparing DNA/DODAC:MO and DNA/DODAC:MO:DC-Chol lipoplexes, at charge ratios (+/-) 3.0 and 4.0, mean size ranges from 126 to 177 nm at charge ratio (+/-) 3.0 and from 118 to 193 nm at charge ratio (+/-) 4.0. As to the DNA/DODAC:MO:DC-Chol complexes, it seems that the higher the MO content, the lower is the charge ratio (+/-) at which this increase in size occurs. The formulation that seems to form smaller complexes is DODAC:MO:DC-Chol (5:4:1). This is also the formulation with the highest MO content.

1.3 Effect of NaCl in liposome and lipoplex mean size and PDI

In order to verify the effect on mean size and PDI of liposomes and lipoplexes in the presence of salts, DODAB/C:MO (2:1) and DODAC:MO:DC-Chol liposomes prepared by film hydration followed by extrution and lipoplexes prepared with salmon sperm DNA and DODAB/C:MO (2:1) and DODAC;MO:DC-Chol (6:3:1, 5:4:1, 4:1:1) liposomes were exposed to different concentrations of NaCl. The mean size and PDI for all the formulations registered upon this exposure are shown in figures 18-20.

1.3.1 Liposomes

The exposure of the liposomes to different concentrations of NaCl seems to influence the mean size of the population. A slight decrease in the mean size of the liposomes can be observed when these are exposed to a gradual increase in NaCl concentration. This is observed for all formulations except for DODAB:MO (2:1), where the liposomes mean size decreases up to 10 mM of NaCl and then suffers a great increase when exposed to higher concentrations of NaCl (Figure 18 (A)).

The inclusion of DC-Chol in DODAC:MO liposomes does not seem to affect the their mean size with increase concentration of salt.

The differences found in the mean size behavior of DODAB/C:MO liposomes might be explained by the different counter-ion. Indeed, Lopes and colleagues (Lopes *et al.*, 2008) have reported that ionic strength is one of the conditions that causes the vesicle size to vary. DODAB has Bromide as counter ion, and the hydration of DODAB:MO liposomes occurs differently according the amount of chloride present in solution. At some point there might even be counter ion exchange. Since chloride is the counterion found in DODAC, the presence of different amounts of chloride in solution has a milder effect on DODAC:MO liposomes.

The PDI values remain relatively constant for all concentrations for all formulations except, again, for the DODAB:MO (2:1) vesicles. The PDI values for this formulation are higher than the PDI values observed for all the other formulations, even in the absence of the salt. Its value increases with the increase of NaCl concentration, at first only slightly, but then after 10 mM of NaCl, its increase is markedly higher (Figure 18 (B)).

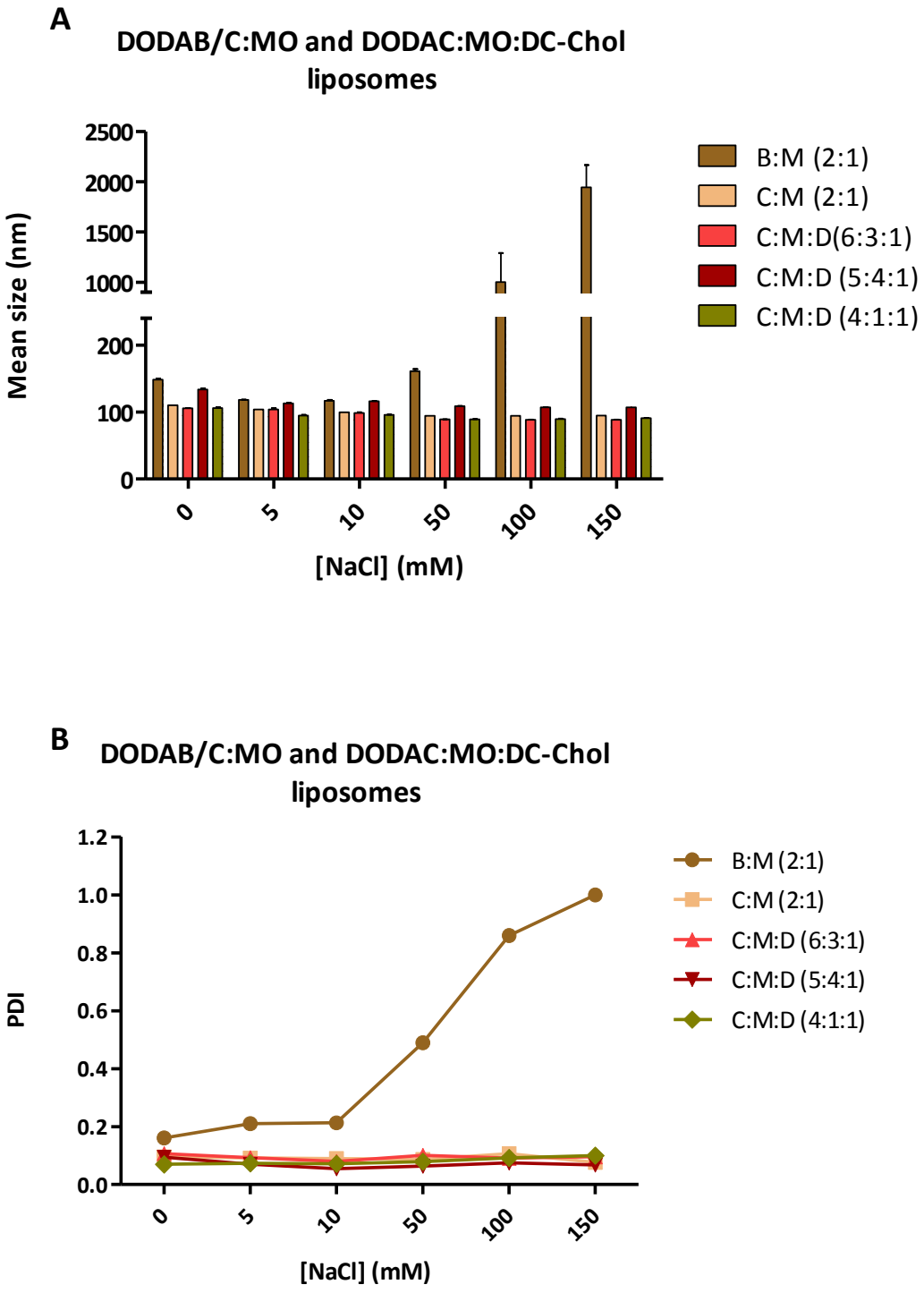


Figure 18. Mean size (A) and polidispersity index (B) of DODAB/C:MO (2:1) and DODAC:MO:DC-Chol (6:3:1, 5:4:1 and 4:1:1) liposomes produced by film hidration followed by extrusion and incubated with different concentrations of NaCl. B:M (2:1) – DODAB:MO liposomes with 2:1 ratio; C:M (2:1) – DODAC:MO liposomes with 2:1 ratio; C:M:D (6:3:1) – DODAC:MO:DC-Chol liposomes with 6:3:1 ratio; C:M:D (5:4:1) – DODAC:MO:DC-Chol liposomes with 5:4:1 ratio; C:M:D (4:1:1) – DODAC:MO:DC-Chol liposomes with 4:1:1 ratio.

1.3.2 Lipoplexes

Figure 19 shows the effect of increasing concentarions of sal in the mean size when the lipoplexes at CR (+/-) 4.0 are exposed to increasing concentrations of salt.

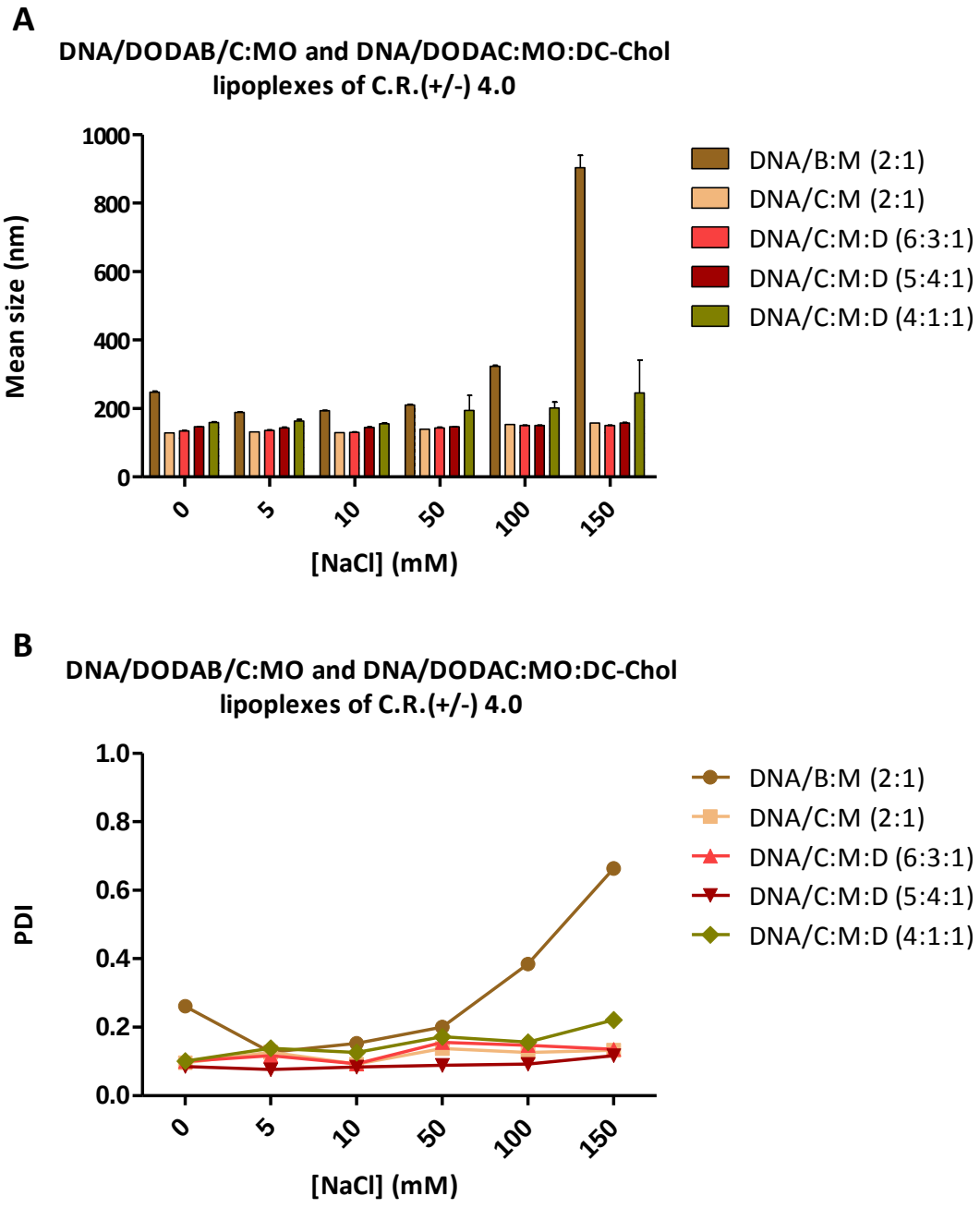


Figure 19. Mean size (A) and polidisperdity index (B) of DNA/DODAB/C:MO (2:1) and DNA/DODAC:MO:DC-Chol (6:3:1, 5:4:1 and 4:1:1) lipoplexes at charge ratio (+/-) 4.0 incubated with different concentrations of NaCl. DNA/B:M (2:1) – DNA/DODAB:MO (2:1) lipoplexes; DNA/C:M (2:1) – DODAC:MO (2:1) lipoplexes; DNA/C:M:D (6:3:1) – DNA/DODAC:MO:DC-Chol (6:3:1) lipoplexes; DNA/C:M:D (5:4:1) – DNA/DODAC:MO:DC-Chol (5:4:1) lipoplexes; DNA/C:M:D (4:1:1) – DNA/DODAC:MO:DC-Chol (4:1:1) lipoplexes.

When the lipoplexes at this charge ratio (+/-) are exposed to increasing concentrations of salt, the mean size of the complexes seems to increase as well, even if it is only slightly for the DNA/DODAC:MO (2:1) and DNA/DODAC:MO:DC-Chol (6:3:1, 5:4:1 and 4:1:1) lipoplexes. The DNA/DODAB:MO (2:1) complexes seem to have a slight increase in the mean size until the NaCl concentration of 10mM is reached. For NaCl concentrations of 50, 100 and 150 mM, the mean size of the complexes suffers a great increase (figure 19 (A)). The same can be verified on the PDI of the lipoplexes. The PDI values of the DNA/DODAC:MO (2:1) and DNA/DODAC:MO:DC-Chol (6:3:1; 5:4:1 and 4:1:1) lipoplexes doesn't suffer significant changes. However, just like the mean size, the PDI of DNA/DODAB:MO (2:1) complexes increases when the NaCl concentration is increased and it can be observed that the highest PDI values registered at charge ratio (+/-) 4.0 belong to DNA/DODAB:MO (2:1) complexes (figure 19 (B)).

When comparing DNA/DODAB:MO and DNA/DODAC:MO lipoplexes, DNA/DODAC:MO lipoplexes seem to be more resistant to changes in mean size when incubated in NaCl. Again, this might be explained by the role played by the conterion in the liposomes used to produce the lipoplexes.

The inclusion of DC-Chol in the liposomal formulation seems to have little effect on the mean size alteration of the lipoplexes produced with these liposomes.

These same measurements were performed in the same conditions to lipoplexes of charge ratio (+/-) 3.0 (data not shown). The lipoplexes prepared at the charge ratio (+/-) 4.0, seem to be more resistant to the increase of salt. This behavior may be due to the fact that at this charge ratio the most DNA is complexed.

The DNA/DODAC:MO lipoplexes at charge ratio (+/-) 4.0, lipoplexes are resistant to the presence of NaCl than DNA/DODAB:MO. Since chloride is present in solution, the DNA/DODAC based lipoplexes don't suffer great perturbation in terms of mean size given that chloride is the counter ion present in DODAC. On the other hand, DODAB has a different counter ion, so the differences in mean size are more noticeable.

1.4 Effect of Serum in liposome and lipoplex mean size and PDI

Interaction of serum components is bound to have an effect in the size of particles. This might influence the way the particles interact with the cellular membrane as well as the uptake pathway through which they enter the cell. Measuring particle size, it could be concluded that different amounts of serum will have different effects on particle size and PDI as well.

In the absence of serum (figure 20), the liposomes present a narrow distribution, as it is to be expected of extruded vesicles. Since they form a homogeneous solution, the PDI value is below 0.1, and this is valid for all formulations. As to the lipoplexes, they presented a mean size distribution which ranged from approximately 140nm to 196nm and the PDI from 0.098 to 0.261 and only one population was detected. The formulations that presented the lowest values of PDI were also the ones with a smaller mean size.

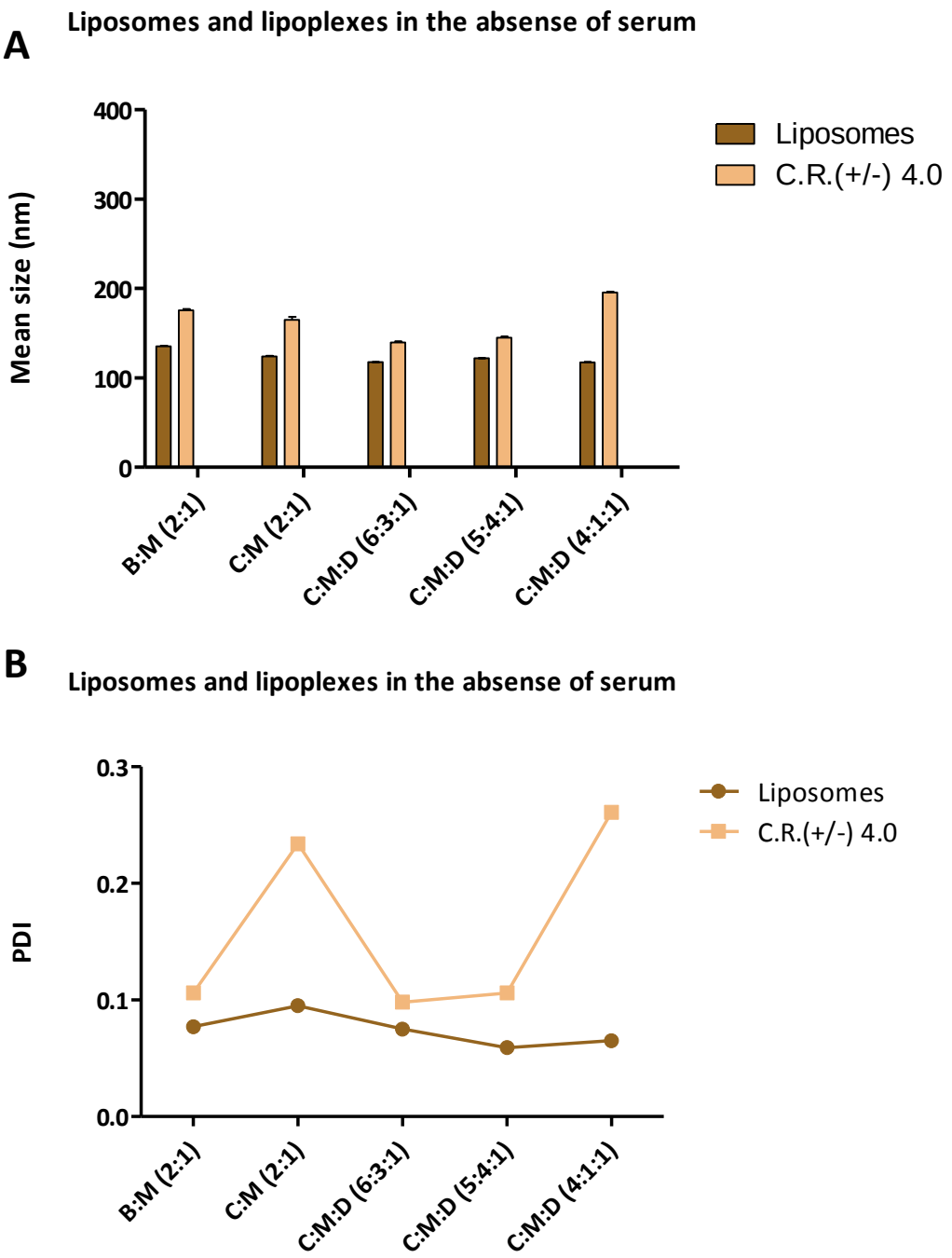


Figure 20. Mean size (A) and polidispersity index (B) of DODA(X):MO (2:1) and DODAC:MO:DC-Chol liposomes (6:3:1, 5:4:1 and 4:1:1) and DNA/DODA(X):MO (2:1) and DNA/DODAC:MO:DC-Chol (6:3:1, 5:4:1 and 4:1:1) lipoplexes at charge ratio (+/-) 4.0 in the absence of serum. B:M (2:1) – DODAB:MO liposomes with 2:1 ratio; C:M (2:1) – DODAC:MO liposomes with 2:1 ratio; C:M:D (6:3:1) – DODAC:MO:DC-Chol liposomes with 6:3:1 ratio; C:M:D (5:4:1) – DODAC:MO:DC-Chol liposomes with 5:4:1 ratio; C:M:D (4:1:1) – DODAC:MO:DC-Chol liposomes with 4:1:1 ratio.

1.4.1 Liposomes

In figure 21 and 22 the effects on the mean size and PDI of DODAB/C:MO and DODAC:MO:DC-Chol liposomes exposed to 30 and 80% of bovine serum.

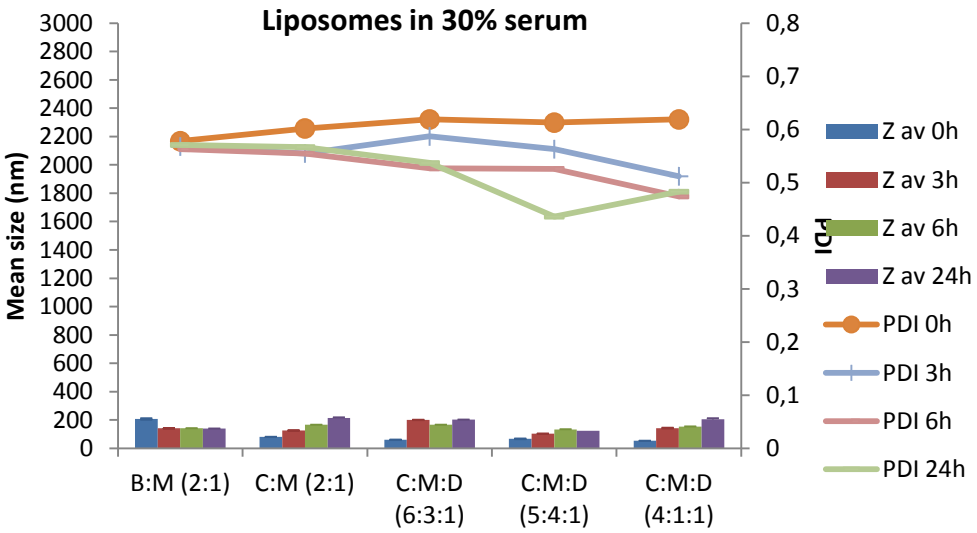


Figure 21. Mean size and polidispersity index (PDI) of DODAB/C:MO (2:1) and DODAC:MO:DC-CHOL (6:3:1, 5:4:1 and 4:1:1) liposomes incubated with 30% bovine serum for 24h. B:M (2:1) – DODAB:MO liposomes with 2:1 ratio; C:M (2:1) – DODAC:MO liposomes with 2:1 ratio; C:M:D (6:3:1) – DODAC:MO:DC-Chol liposomes with 6:3:1 ratio; C:M:D (5:4:1) – DODAC:MO:DC-Chol liposomes with 5:4:1 ratio; C:M:D (4:1:1) – DODAC:MO:DC-Chol liposomes with 4:1:1 ratio.

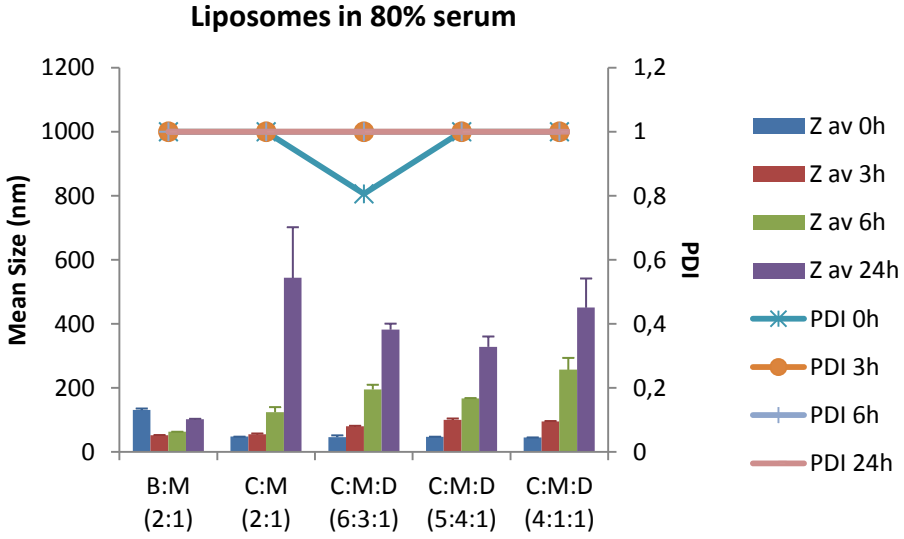


Figure 22. Mean size and polidispersity index (PDI) of DODAB/C:MO (2:1) and DODAC:MO:DC-CHOL (6:3:1, 5:4:1 and 4:1:1) liposomes incubated with 80% bovine serum for 24h. B:M (2:1) – DODAB:MO liposomes with 2:1 ratio; C:M (2:1) – DODAC:MO liposomes with 2:1 ratio; C:M:D (6:3:1) – DODAC:MO:DC-Chol liposomes with 6:3:1 ratio; C:M:D (5:4:1) – DODAC:MO:DC-Chol liposomes with 5:4:1 ratio; C:M:D (4:1:1) – DODAC:MO:DC-Chol liposomes with 4:1:1 ratio.

The incubation of liposomes in 30% serum resulted in a slight decrease in the mean size at the timepoint 0h (the measurement was performed right after the particles incubation with serum) but as time passed, the mean size value of DODAC based liposomes increased. For DODAB:MO (2:1) liposomes the results were quite the opposite. These liposomes suffered a slight increase at time point 0h, when compared to the measurements performed in the absence of serum, but their mean size decreased after 6h. In 80% of serum, the effect in mean size appears to be the same for all formulations, although the increase in mean size is higher, especially after 24h of incubation. Moreover PDI values increase for both DODAB/C:MO and DODAC:MO:DC-Chol liposomes, but remains more or less constant throughout time. For liposomes incubated with 30% serum PDI rises to values above 0.5 but for liposomes incubated with 80% serum this increase in PDI is higher, reaching values of 1.0.

DODAB:MO (2:1) liposomes appear to be more resistant to the effect of serum than DODAC:MO (2:1) liposomes. The inclusion of DC-Chol in the formulation appears not to influence the resistance of DODAC:MO based liposomes.

1.4.2 Lipoplexes

The effect of serum on lipoplexes was tested by incubation of DNA/DODAB/C:MO and DNA/DODAC:MO:DC-Chol lipoplexes at charge ratio (+/-) 4.0 in 30 and 80% of bovine serum. The results are displayed in figures 23 and 24.

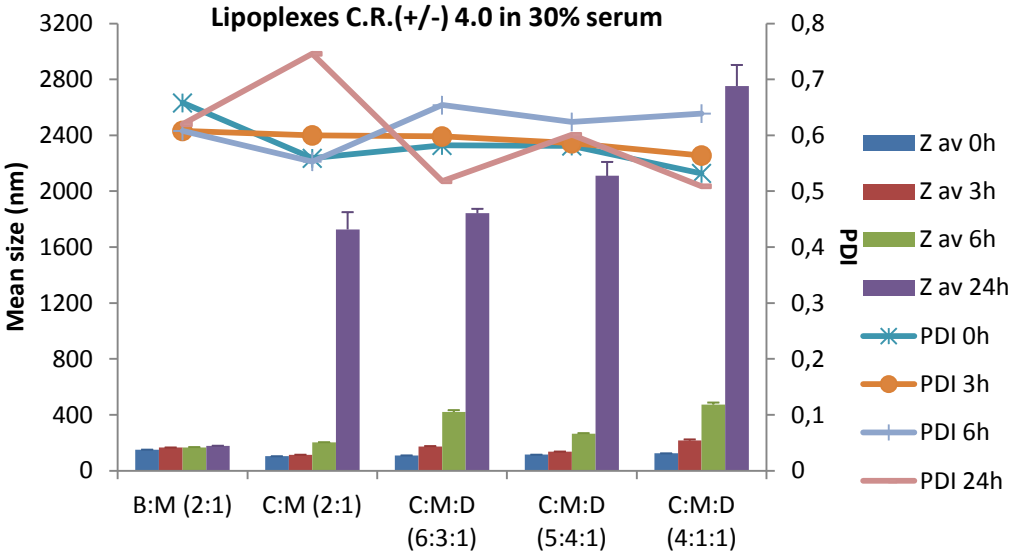


Figure 23. Mean size and polidispersity index of DNA/DODAB/C:MO (2:1) and DNA/DODAC:MO:DC-Chol (6:3:1, 5:4:1 and 4:1:1) lipoplexes at charge ratios (+/-) 4.0 incubated with 30% bovine serum for 24h. DNA/B:M (2:1) – DNA/DODAB:MO (2:1) lipoplexes; DNA/C:M (2:1) – DODAC:MO (2:1) lipoplexes; DNA/C:M:D (6:3:1) – DNA/DODAC:MO:DC-Chol (6:3:1) lipoplexes; DNA/C:M:D (5:4:1) – DNA/DODAC:MO:DC-Chol (5:4:1) lipoplexes; DNA/C:M:D (4:1:1) – DNA/DODAC:MO:DC-Chol (4:1:1) lipoplexes.

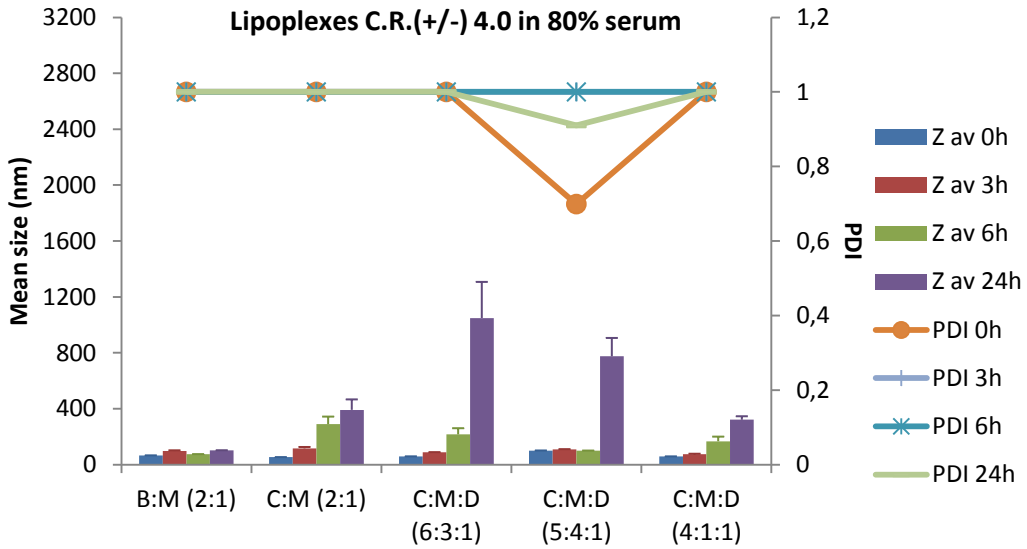


Figure 24. Mean size and polidispersity index of DNA/DODAB/C:MO (2:1) and DNA/DODAC:MO:DC-Chol (6:3:1, 5:4:1 and 4:1:1) lipoplexes at charge ratios (+/-) 4.0 incubated with 80% bovine serum for 24h. DNA/B:M (2:1) – DNA/DODAB:MO (2:1) lipoplexes; DNA/C:M (2:1) – DODAC:MO (2:1) lipoplexes; DNA/C:M:D (6:3:1) – DNA/DODAC:MO:DC-Chol (6:3:1) lipoplexes; DNA/C:M:D (5:4:1) – DNA/DODAC:MO:DC-Chol (5:4:1) lipoplexes; DNA/C:M:D (4:1:1) – DNA/DODAC:MO:DC-Chol (4:1:1) lipoplexes.

In figure 23, it is visible that DNA/DODAB:MO lipoplexes at CR(+/-) 4 look very stable in 30% serum during 24h. On the other hand, DNA/DODAC:MO lipoplexes display a feeble increase in mean size for the first 6h and then suffer a huge increase after 24h. These results appear to go along with the results obtained for DNA/DODAC:MO:DC-Chol lipoplexes. PDI values are also higher than the values registered for the lipoplexes in the absence of serum for all the tested formulations. The DODAB:MO liposomes seem to manifest relatively constant values of PDI along the 24h. DODAC:MO liposomes maintain roughly the same values of PDI for the first 6h and then suffer an increment at 24h. The DODAC:MO:DC-Chol liposomes PDI values go along with the values obtained for DODAC:MO liposomes for the first 3h. After 6h the values seem to rise and then lower again at 24h relatively to the values registered for DNA/DODAC:MO liposomes.

When incubated with 80 % of serum, the DNA/DODAB:MO (2:1) lipoplexes appear to maintain a constant mean size for 24h. DNA/DODAC:MO (2:1) and DNA/DODAC:MO:DC-Col lipoplexes seem to display an increase the mean size although the DODAC:MO:DC-Chol 5:4:1 and 4:1:1 size increment is less than the one displayed for DODAC:MO:DC-Chol 6:3:1 formulation (Figure 24). Furthermore the mean size reached by the lipoplexes incubated with 80% serum was not as high as the mean size reached when incubated with 30% serum. The PDI also suffered a great increase, reaching the value of 1 right after the incubation, except for the DNA/DODAC:MO:DC-Chol (5:4:1) formulation that only displayed this PDI value after 24h.

The results for lipoplexes at charge ratio (+/-) 4.0 are in accordance with the results for lipoplexes of charge ratio (+/-) 3.0. (data not shown).

It can be observed that the mean size and PDI of DNA/DODAB:MO (2:1) lipoplexes are more resistance to serum compared with the DNA/DODAC:MO (2:1) lipoplexes.

Serum is composed of many proteins with smaller sizes and this influences the mean size. Actually, the measurements of the particles incubated with serum recorded the presence of three populations and the main population presents higher values of size when compared to the values registered in the absence of serum (see Appendix 3). This might be due to the aggregation of smaller components of serum around the

liposomes and lipoplexes. Also, the percentage of particles of the main population decreases throughout time as the percentage of smaller populations increases (see Appendix 3). These smaller particles also increase in size as time goes by, which leads again to the possibility of particle aggregation, both around liposomes and lipoplexes and also between serum components and it is an effect observed for all liposomal formulations. This might explain the lower values of mean size for lipoplexes incubated with 80% of serum, since the percentage of smaller particles that compose bovine serum is larger.

It is interesting to note that DNA/DODAB/C:MO lipoplexes seem to be affect differently by serum components in terms of mean size. The different counter-ion appears to play a role in this. Also, DNA/DODAC:MO:DC-Chol lipoplexes show comparable results in terms of mean size alteration comparable to the ones observed in DNA/DODAC:MO lipoplexes in the first 6h. After 24h of incubation, DNA/DODAC:MO:DC-Chol liposomes have an higher increase in size relatively to DNA/DODAC:MO liposomes.

The aggregation promoted by serum might be due to negatively charged serum proteins interacting with positively charged lipoplexes, coating their surface. Another possibility is the formation of “protein corona” in proteins resulting from the competition for the cationic membrane surface, which may force lipid vesicles to aggregate as a consequence of intermembrane repulsive barrier reduction (Yang *et al*., 2012).

It is usually observed low transfection efficiency in medium containing serum, partially due to the possibility of lipoplex instability caused by serum components. It has been reported in literature that the inclusion of cholesterol or cholesterol derivates, such as DC-Chol, into the liposomal formulation, reduces permeability to solutes and increases the packing parameter of phospholipids and that this increase can help to preserve liposomal stability in serum. Experiments performed with DC-Chol-DOPE liposomes and DNA/DC-Chol-DOPE lipoplexes showed that there was an increase in transfection efficiency in the presence of serum (Caracciolo *et al*., 2010). Both liposomes and lipoplexes presented an increase in size when incubated in 50% of serum. The authors suggested that the surface adsorption of protein corona results in a switch on the entry pathway into the cell. Instead of entering through a clathrin-

dependent pathway, the lipoplexes should enter through caveolae-mediated mechanisms (Caracciolo *et al*., 2010). Indeed, the inclution of DC-Chol into the liposomal formulation seemed to result in better transfection efficiency, as it will be discussed later in this study. However, further studies are needed to comprehend serum interaction with lipoplexes and how that affects transfection efficiency.

2. *ζ -potencial* assays

2.1 ζ -*potencial of liposomes*

The ζ –potencial of liposomes seems to vary according to the liposomal formulation and with the method of liposomal preparation, as it can be seen on figure 25. For DODAB based liposomes, the values of zeta potencial obtained for the extruded liposomes don't seem to vary significantly with the inclusion of DC-Chol in the formulation. However, for liposomes produced by ethanolic injection, the DODAB:MO:DC-Chol (6:3:1) presents a lower value of ζ –potencial when compared with the other DODAB based liposomes.

DODAC based liposomes obtained by ethanolic injection seem to display an increase in the ζ –potencial values with the inclusion of DC-Cholesterol. On the other hand, extruded vesicles seem to exhibit an opposite behavior. DODAC:MO:DC-Chol extruded vesicles present slightly lower values of ζ –potencial when compared to DODAC:MO (2:1) vesicles.

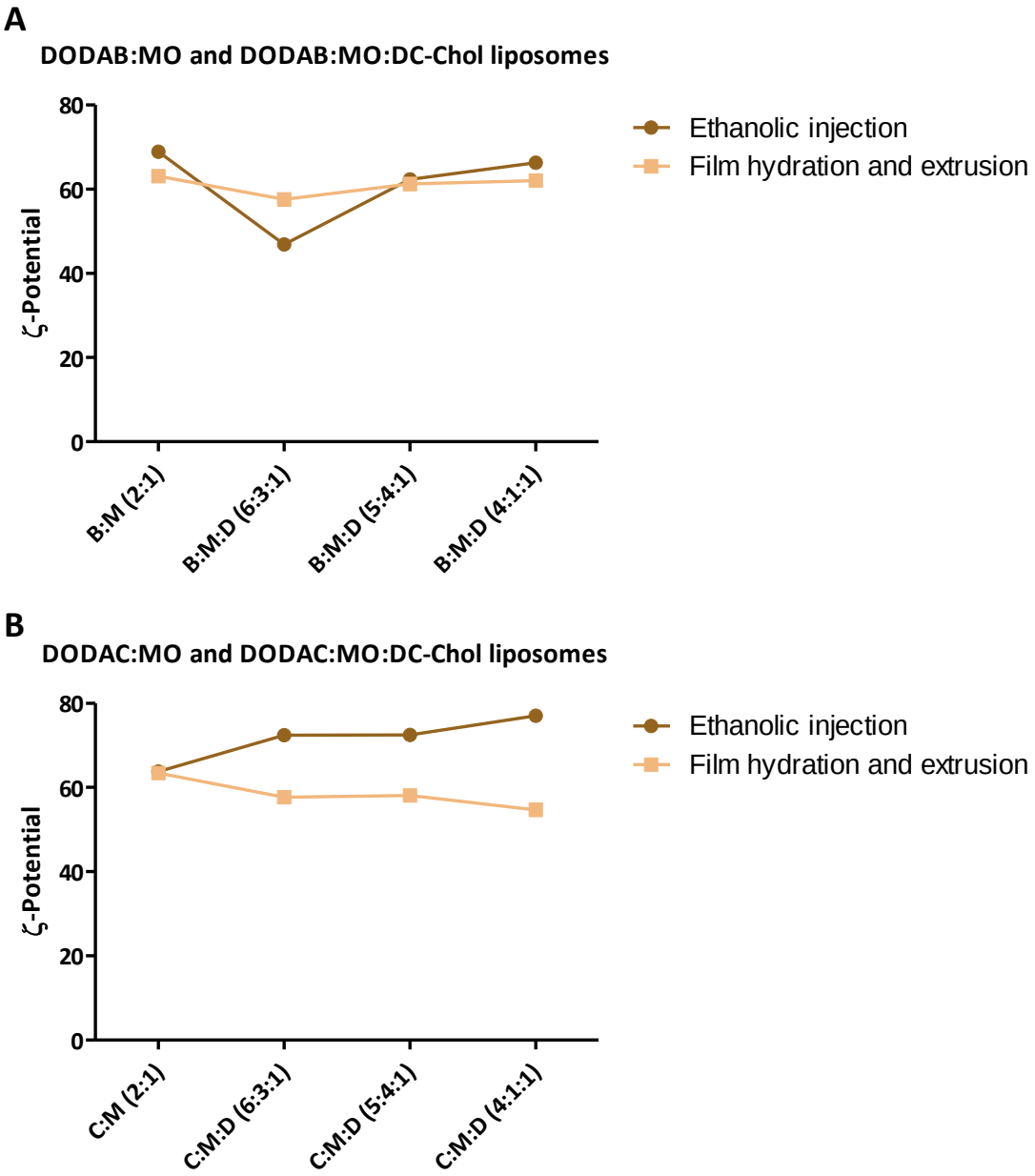


Figure 25. ζ-potential of: (A) DODAB:MO (2:1) and DODAB:MO:DC-CHOL (6:3:1, 5:4:1 and 4:1:1) liposomes produced by ethanolic injection and by film hydration followed by extrusion; (B) DODAC:MO (2:1) and DODAC:MO:DC-CHOL (6:3:1, 5:4:1 and 4:1:1) liposomes produced by ethanolic injection and by film hydration followed by extrusion. B:M (2:1) – DODAB:MO liposomes with 2:1 ratio; C:M (2:1) – DODAC:MO liposomes with 2:1 ratio; B:M:D (6:3:1) – DODAB:MO:DC-Chol liposomes with 6:3:1 ratio; C:M:D (6:3:1) – DODAC:MO:DC-Chol liposomes with 6:3:1 ratio; B:M:D (5:4:1) – DODAB:MO:DC-Chol liposomes with 5:4:1 ratio; C:M:D (5:4:1) – DODAC:MO:DC-Chol liposomes with 5:4:1 ratio; B:M:D (4:1:1) – DODAB:MO:DC-Chol liposomes with 4:1:1 ratio; C:M:D (4:1:1) – DODAC:MO:DC-Chol liposomes with 4:1:1 ratio.

This might be a consequence of the method of liposome preparation, since ethanolic injection liposomes are very hard to be reproduced and parameters like

liposome size cannot be controlled. The size of the particles might be one parameter that explains the observed differences.

2.2 ζ-potencial of lipoplexes

The ζ-potencial of DNA/DODAB/C:MO (2:1) and DNA/DODAC:MO:DC-Chol lipoplexes prepared at different charge ratios was measured. The results are represented in figure 26.

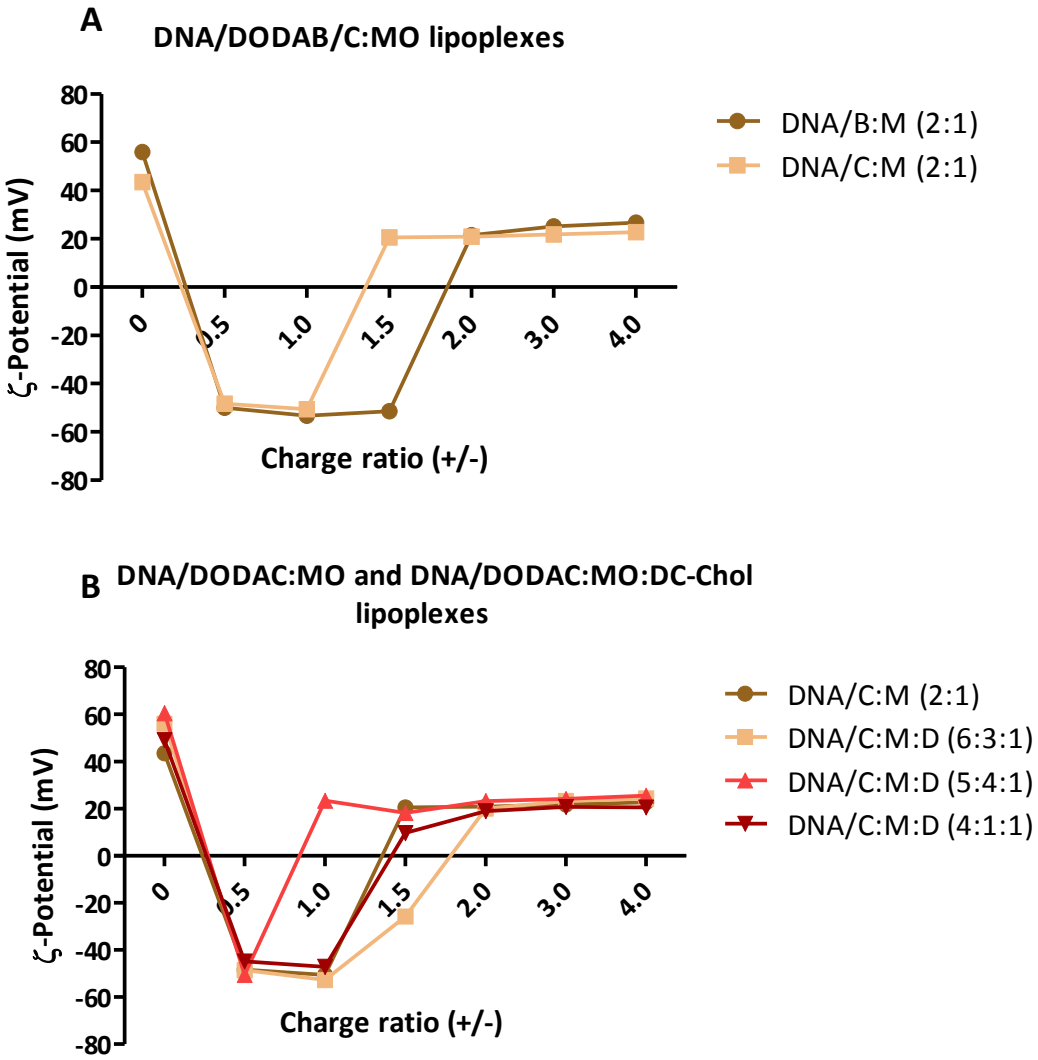


Figure 26. ζ-potencial: (A) DNA/DODAB/C:MO (2:1) lipoplexes; (B) DNA/DODAC:MO (2:1) and DNA/DODAC:MO:DC-Chol (6:3:1, 5:4:1 and 4:1:1) lipoplexes at different charge ratios (+/-). DNA/B:M (2:1) – DNA/DODAB:MO (2:1) lipoplexes; DNA/C:M (2:1) – DODAC:MO (2:1) lipoplexes; DNA/C:M:D (6:3:1) – DNA/DODAC:MO:DC-Chol (6:3:1) lipoplexes; DNA/C:M:D (5:4:1) – DNA/DODAC:MO:DC-Chol (5:4:1) lipoplexes; DNA/C:M:D (4:1:1) – DNA/DODAC:MO:DC-Chol (4:1:1) lipoplexes.

Pure cationic liposomes exhibit a positive ζ- potential 43-60mV, depending on the lipid formulation. The formulation that presents the highest value is DODAC:MO:DC-Chol (5:4:1) and the one that presents the lowest value is DODAC:MO (2:1). Pure DNA presents negative ζ- potential values (data not shown). At lower charge ratios (+/-) like 0.5, the ζ- potential of the complexes is negative for all formulations. As the charge ratio (+/-) increases, the ζ- potential also increases until it reaches an isoelectric point. The charge ratio (+/-) at which the isoelectric point is reached differs according to the formulation and coincides with the great increase in mean size and PDI of the complexes mentioned previously in this study. After the neutralization point, the ζ- potential keeps increasing until charge ratio (+/-) 2.0 where it stabilizes and suffers only a slight increase in charge ratios (+/-) 3.0 and 4.0. At charge ratio (+/-) 4.0 the value associated with the ζ- potential is around 20-26 mV (Figure 26). The DNA/DODAC:MO (2:1) lipoplexes seem to reach the isoelectric point at a lower charge ratio (+/-) the DNA/DODAB:MO (2:1) lipoplexes. As to the DNA/DODAC:MO:DC-Chol lipoplexes, the higher amount of MO associated with DC-Chol in the formulation (5:4:1) seems to help in DNA complexation, since it reaches the isoelectric point at the lowest charge ratio(+/-) when compared with all the other formulations. This suggests that the complexation is different and dependent of the liposomal formulation, which also suggests structural variations between different formulations.

2.3 Effect of NaCl in liposome and lipoplex ζ-potencial

The ζ-potential of liposomes varies with each formulation in the absence of NaCl as previously stated. Previously in this study, the effects of NaCl in the mean size and PDI were assessed. In order to assess the effect of NaCl in the particles ζ-potential measurements of DODAB/C:MO and DODAC:MO:DC-Chol liposomes and DNA/DODAB/C:MO:DC-Chol and DNA/DODAC:MO:DC-Chol lipoplexes ζ-potential incubated with NaCl were performed.

2.3.1 Liposomes

Figure 27 shows that upon increasing NaCl concentration up to 10 mM, the ζ-potential suffers a slight increase except for the formulation (4:1:1), which suffers a slight decrease. At 10mM, the formulation (4:1:1) reaches a plateau, while the other formulations present a decrease in ζ-potential with the increase of NaCl concentration up to 150mM of NaCl. The observed values of ζ-potential at 150mM are lower the registered values of ζ-potential in the absence of NaCl. DODAC:MO (2:1) liposomes display lower values of ζ-potential than DODAB:MO (2:1) liposomes up to NaCl concentration of 10 mM. With increasing concentration of NaCl the opposite effect is observed. DODAC:MO:DC-Chol 6:3:1 and 5:4:1 formulations present slightly higher values than DODAC:MO (2:1). The DODAC:MO:DC-Chol (4:1:1) liposomes seem to be the exception.

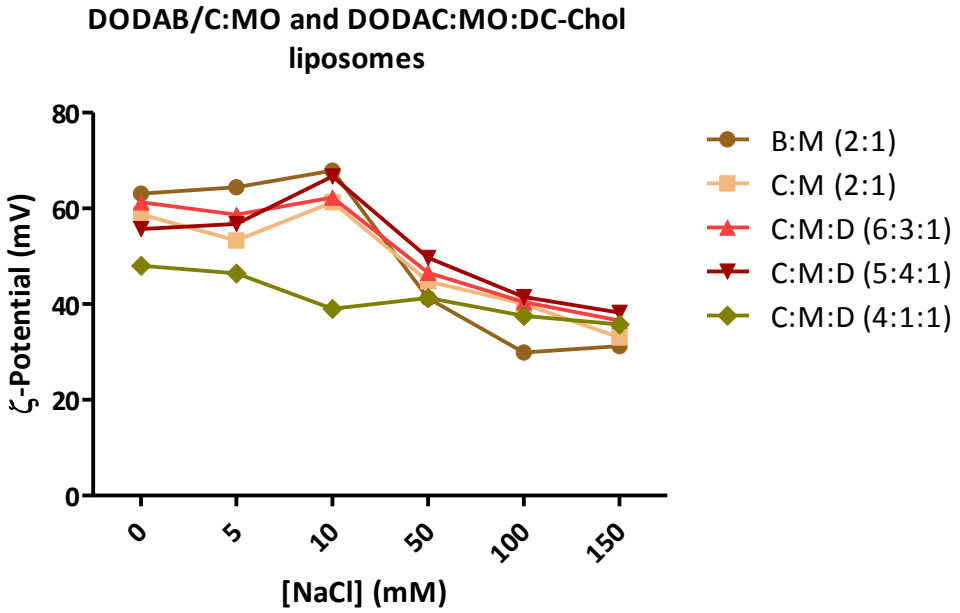


Figure 27. ζ-potencial of DODAB/C:MO (2:1) and DODAB/C:MO:DC-CHOL (6:3:1, 5:4:1 and 4:1:1) liposomes produced by ethanolic injection and incubated with different concentrations of NaCl. B:M (2:1) – DODAB:MO liposomes with 2:1 ratio; C:M (2:1) – DODAC:MO liposomes with 2:1 ratio; C:M:D (6:3:1) – DODAC:MO:DC-Chol liposomes with 6:3:1 ratio; C:M:D (5:4:1) – DODAC:MO:DC-Chol liposomes with 5:4:1 ratio; C:M:D (4:1:1) – DODAC:MO:DC-Chol liposomes with 4:1:1 ratio.

2.3.2 Lipoplexes

The effects on the ζ-potential of lipoplexes of charge ratio (+/-) 4.0 incubated with NaCl at different concentrations is represented in figure 28.

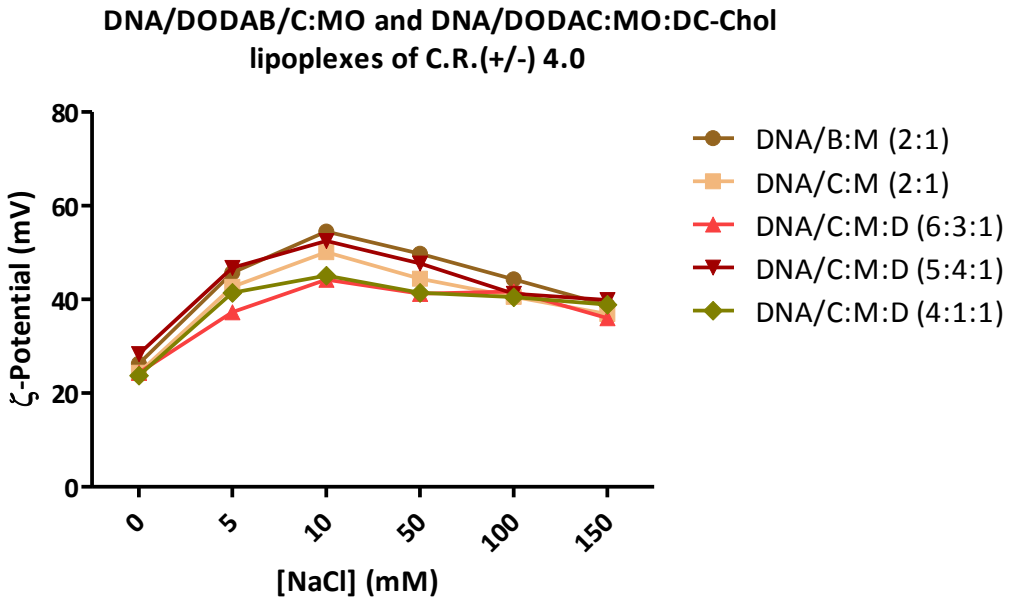


Figure 28. ζ-potencial of DNA/DODAB/C:MO (2:1), DNA/DODAC:MO (2:1) and DNA/DODAC:MO:DC-Chol (6:3:1, 5:4:1 and 4:1:1) lipoplexes at charge ratio (+/-) 4.0 incubated with different concentrations of NaCl. DNA/B:M (2:1) – DNA/DODAB:MO (2:1) lipoplexes; DNA/C:M (2:1) – DODAC:MO (2:1) lipoplexes; DNA/C:M:D (6:3:1) – DNA/DODAC:MO:DC-Chol (6:3:1) lipoplexes; DNA/C:M:D (5:4:1) – DNA/DODAC:MO:DC-Chol (5:4:1) lipoplexes; DNA/C:M:D (4:1:1) – DNA/DODAC:MO:DC-Chol (4:1:1) lipoplexes.

The ζ-potential increases until a concentration of 10mM of NaCl is reached and there it presents its higher value. It then starts to decrease as the NaCl increases. At 150 mM, the maximum concentration of NaCl tested, the ζ-potential of the lipoplexes is still higher than the ζ-potential of the lipoplexes in the absence of NaCl. The ζ-potential of lipoplexes seems to display the same tendency on both charge ratios (+/-) 3.0 and 4.0 (data not shown).

3. Fluorescence Ressonant Energy Transfer (FRET) assays

The Fluorescence Resonance Energy Transfer (FRET) was used to monitor the complexation process of DNA. BOBO-1 was used to labeled DNA acting as a donor and rho-PE used to labeled liposomes acting as acceptor. This energy transfer was monitored for DNA/DODA(X):MO (2:1) and DNA/DODAC:MO:DC-Chol (6:3:1, 5:4:1, 4:1:1) lipoplexes. It was observed a decrease in the fluorescence emission of BOBO-1 and the corresponding increase of fluorescence emission of rho-PE, indicating that an energy transfer between the two probes occurs as the charge ratio (+/-) increases. Figure 29 e Figure 30 presents the specral behavior for DNA:DODAB:MO and DNA:DODAC:MO).

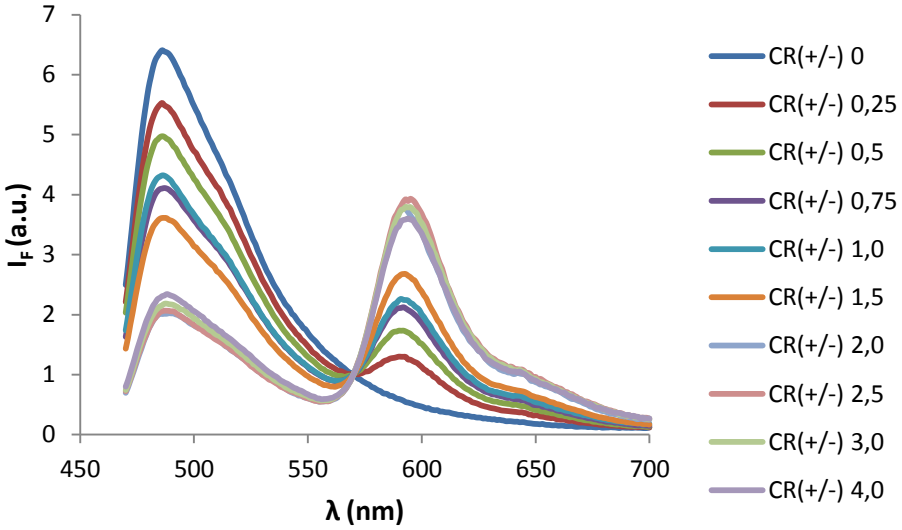


Figure 29. Fluorescence emission spectra of BOBO-1 and rho-PE for DNA/DODAB:MO (2:1) lipoplexes at different charge ratios (+/-).

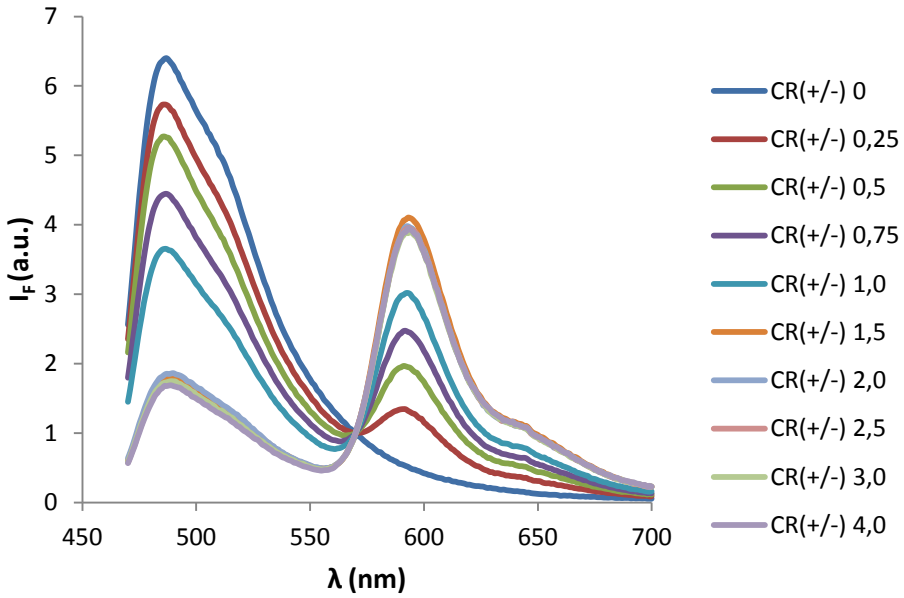


Figure 30. Fluorescence emission spectra of BOBO-1 and rho-PE for DNA/DODAC:MO (2:1) (lipoplexes at different charge ratios (+/-).

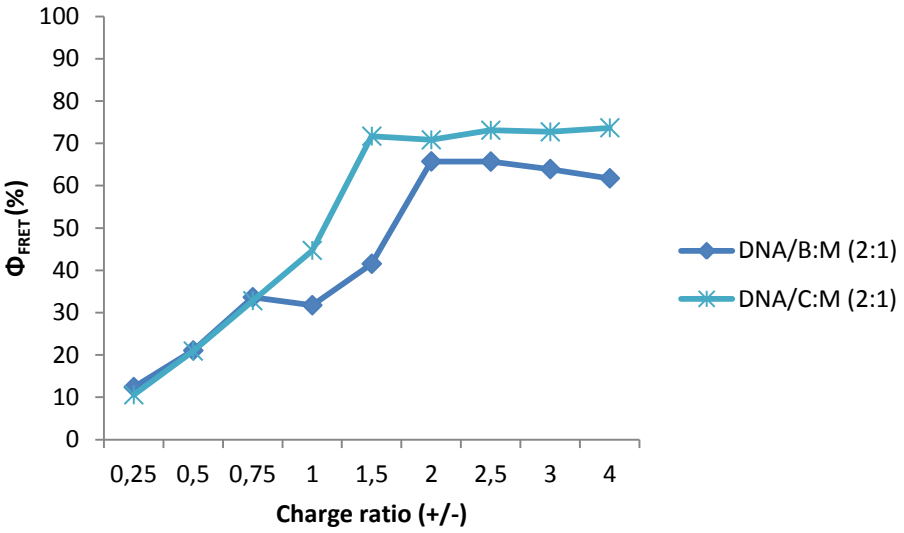


Figure 31. FRET efficiency of DNA/DODAB/:MO (2:1) at different charge ratios (+/-). DNA/B:M (2:1): DNA/DODAB:MO (2:1) lipoplexes; DNA/C:M (2:1): DNA/DODAC:MO (2:1).

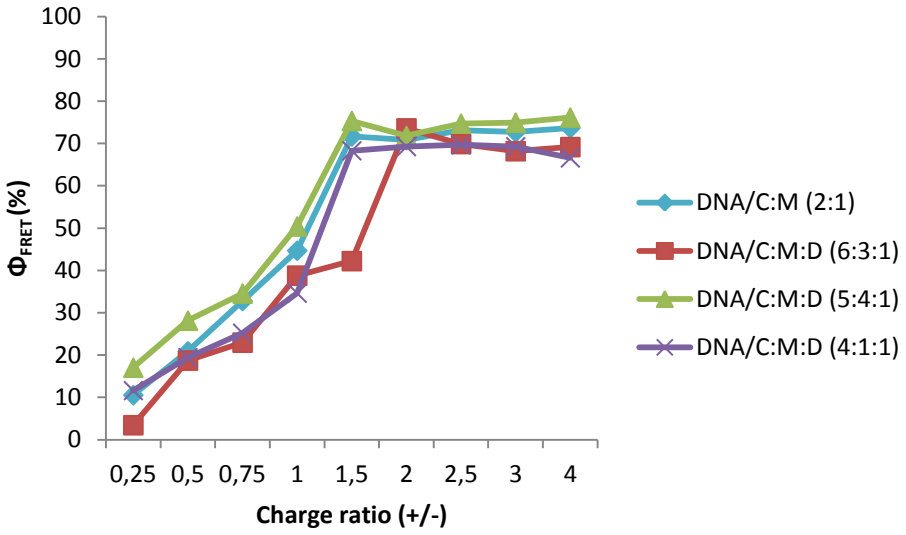


Figure 32. FRET efficiency of DNA/DODAC:MO (2:1) and DNA/DODAC:MO:DC-Chol (ratios 6:3:1, 5:4:1, 4:1:1) at different charge ratios (+/-). DNA/C:M (2:1): DNA/DODAC:MO (2:1) lipoplexes; DNA/C:M:D (6:3:1): DNA/DODAC:MO:DC-Chol (6:3:1) lipoplexes; DNA/C:M:D (5:4:1): DNA/DODAC:MO:DC-Chol (5:4:1) lipoplexes; DNA/C:M:D (4:1:1): DNA/DODAC:MO:DC-Chol (4:1:1) lipoplexes.

In figures 31 and 32, it can be observed the efficiency of FRET (Φ_{FRET}), which reflects the complexation efficiency of the lipoplexes at different charge ratios (+/-). The efficiency increases until a certain charge ratio (+/-) where it reaches a plateau. The DODAC:MO (2:1) lipoplexes reach a plateau at charge ratio (+/-) 1.5 and DODAB:MO (2:1) lipoplexes reach a plateau at charge ratio (+/-) 2.0. DODAC:MO:DC-Chol (5:4:1 and 4:1:1) lipoplexes reach a plateau at the same charge ratio (+/-) as DODAC:MO (2:1) lipoplexes but DODAC:MO:DC-Chol (6:3:1) lipoplexes reach it at charge ratio (+/-) 2.0. These results come in line with the results obtained from DLS and ζ-potential previously reported. The formulations which reach the plateau at charge ratio (+/-) 1.5 are also the formulations that reached the isoelectric point at lower charge ratios (+/-). At charge ratio (+/-) 4.0, the efficiency of FRET follows the order: C:M:C (5:4:1)> C:M (2:1)> C:M:C (6:3:1) > C:M:C (4:1:1) >B:M (2:1).

It seem that chloride has a beneficial effect on DNA complexation on the DODAB/C:MO system as it can be observed when comparing the complexation efficiency of DODAB:MO (2:1) liposomes and DODAC:MO (2:1) liposomes. This might

be due to the size of the counter-ion chloride, which is smaller than Bromide and which might facilitate DNA complexation with liposomes.

The inclusion of DC-Chol doesn't seem to affect greatly the complexation efficiency of the system. It should be noted that the formulation which presents higher efficiency is DODAC:MO:DC-Chol (5:4:1). This is also the formulation with higher amount of MO. In previous studies with DODAB:MO vesicles, it was shown that there was a high dependence of the DNA complexation efficiency and lipoplex structure on the MO content. The fluidizing effect of MO is thought to be the reason for this high dependency since it favours lipid chains mobility (Neves Silva *et al*., 2008).

4. Citotoxicity

The citotoxicity induced by liposomes and lipoplexes was evaluated in two different cell lines. Murine fibroblasts, L929 cell line, were exposed for 48h to DODAB/C:MO (2:1) and DODAB/C:MO DC-Chol (ratios 6:3:1, 5:4:1 and 4:1:1) liposomes prepared by ethanolic injection or by film hydration method followed by extrusion. Human embryonic kidney cells (293T cell line) was also exposed to lipoplexes prepared by different liposomal formulations for 48h as well.

MTT assay can be used to assess cell viability by acting as a marker of the cell metabolic capacity. Assessing the membrane integrity by LDH assay was also a method used to corroborate the results obtained by estimating cell viability through the metabolic capacity in MTT assays. Since only the Extracellular LDH was quantified, the enzymatic activity of this enzyme was calculated from the obtained values. The LDH enzyme is an intracellular enzyme that is released to the extracellular medium when the membrane integrity is compromised.

4.1 DODAB/C:MO:DC-Chol liposomes

Again, the counter ion exchange seems to somehow affect differently cell viability. In figure 33 it is possible to observe that the cell metabolism is more severely by DODAC liposomes compared to DODAB based liposomes. When exposed to a lipid concentration of 50 µg/mL, cell survival is practically null when in contact with liposomes produced by ethanolic injection or for liposomes produced by extrusion. At

a lipid concentration of 5 µg/mL, the liposomes produced by extrusion seem to be a little less toxic than the liposomes produce by ethanolic injection and this seems to be applicable to all formulations. Also, the inclusion of DC-Chol doesn't seem to increase significantly the systems toxicity when compared to DODAC:MO (2:1) liposomes. In fact, the extruded liposomes with (5:4:1) and (4:1:1) formulations seem to be slightly less toxic than the DODAC:MO (2:1) liposomes (Figure 33).

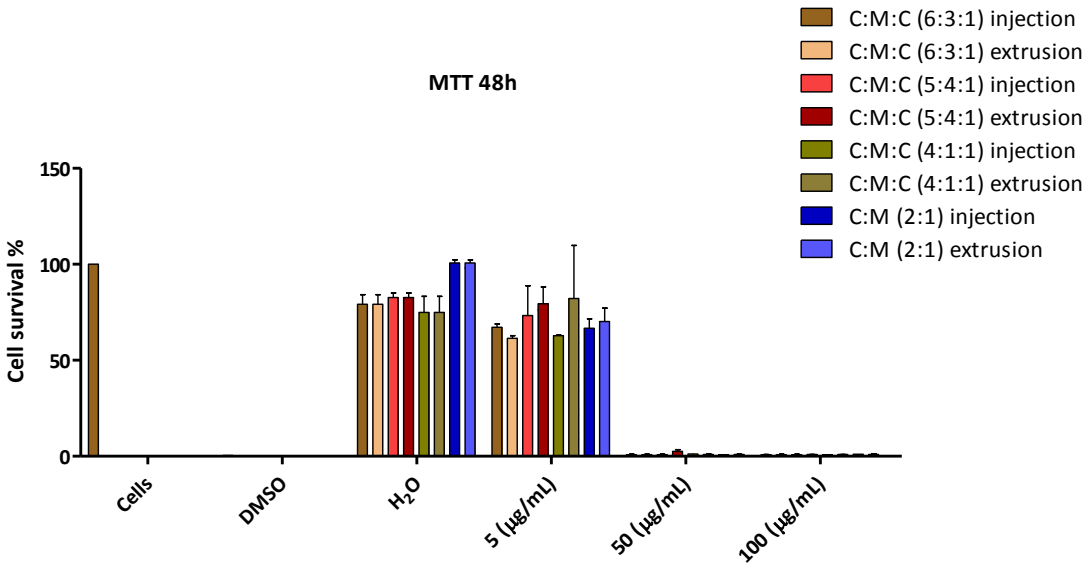


Figure 33. Evaluation of the cytotoxicity (metabolic assay) in L929 cell line induced by induced by varying concentrations of DODAC:MO (2:1) and DODAC:MO:DC-Chol (6:3:1, 5:4:1, 4:1:1) prepared by ethanolic injection and film hydration followed by extrusion after 48h of incubation. Cells: cells alone; Cells: cells alone; DMSO: cells incubated with 30 % DMSO; H2O: cells incubated with the volume of H2O used to incubate de liposomes to a final concentration of 100 µg/mL. The mean (+/−) SD was obtained from two independent experiments.

On the other hand, the DODAB based liposomes seem to be less toxic than the DODAC based liposomes, especially the liposomes produced by extrusion. At a lipid concentration of 50 µg/mL, the viability of cells exposed to liposomes prepared by ethanolic injection is practically null but the cells exposed to this same concentration of extruded liposomes seem to have a survival rate of 50% approximately for the DODAB:MO:DC-Chol liposomes and around 80% survival rate for DODAB:MO (2:1) liposomes (Figure 34).

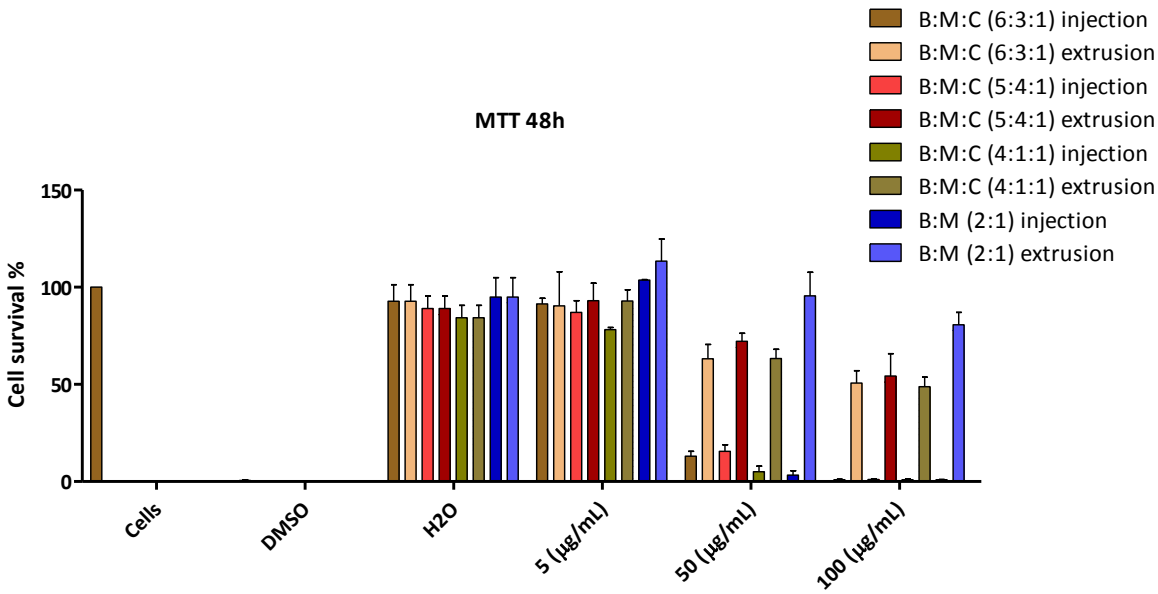


Figure 34. Evaluation of the cytotoxicity (metabolic assay) in L929 cell line induced by induced by varying concentrations of DODAB:MO (2:1) and DODAB:MO:DC-Chol (6:3:1, 5:4:1, 4:1:1) prepared by ethanolic injection and film hydration followed by extrusion after 48h of incubation. Cells: cells alone; Cells: cells alone; DMSO: cells incubated with 30 % DMSO; H2O: cells incubated with the volume of H2O used to incubate de liposomes to a final concentration of 100 µg/mL. The mean (+/−) SD was obtained from two independent experiments.

The liposomes prepared by ethanolic injection seem to be more toxic than the extruded vesicles, a fact that seems to be more noticeable for DODAB based liposomes. This might be related to the preparation system. Ethanol, the organic solvent used in liposome production might not be totally evaporated when in touch with the water. Also, the liposomes obtained by this method are more polydisperse and it is very difficult to reproduce the liposomes with identical sizes by this method since this feature cannot be as controlled as with extrusion, where membranes with a defined pore are used.

Incubation with DODAC based liposomes seems to be associated with higher extracellular activity of the LDH enzyme, which reflects a higher number of cells with compromised membrane integrity. This is in agreement with the MTT results previously shown regarding DODAC based liposomes. Again, the inclusion of DC-Chol appears not to make a great difference in terms of cell viability when compared to DODAC:MO (Figure 35).

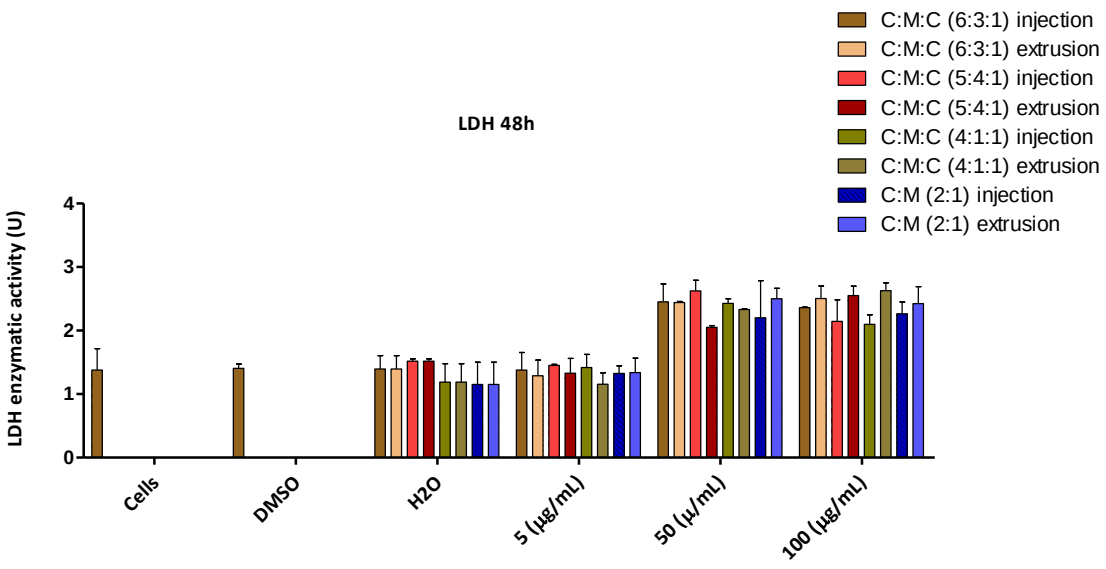


Figure 35. Evaluation of the cytotoxicity (cell membrane integrity) in L929 cell line induced by varying concentrations of DODAC:MO (2:1) and DODAC:MO:DC-Chol (6:3:1, 5:4:1, 4:1:1) after 48h of incubation. Cells: cells alone; DMSO: cells incubated with 30 % DMSO; H2O: cells incubated with the volume of H2O used to incubate de liposomes to a final concentration of 100 µg/mL. The mean (+/−) SD was obtained from two independent experiments.

As to DODAB based liposomes, the extracellular LDH activity upon incubation with liposomes prepared by ethanolic injection is markedly higher at a lipid concentration of 100 µg/mL, which coincides with decreased cell viability registered in the MTT assays. However, the effect observed with the MTT assays regarding the addition of DC-Chol to the liposomal formulation is not so easily evident in the LDH assays. Furthermore, extracellular LDH enzyme activity in cells exposed to extruded liposomes at a concentration of 100 µg/mL is practically the same registered for exposure to lower concentrations of liposomes (Figure 36). This conflicts with the results obtained by the MTT assay, where cell viability decreases to almost 50% in cells exposed to DODAB:MO:DC-Chol extruded liposomes at a lipid concentration of 100 µg/mL. These discrepancies may be explained by the fact that the metabolism and membrane integrity are two different aspects concerning cell viability.

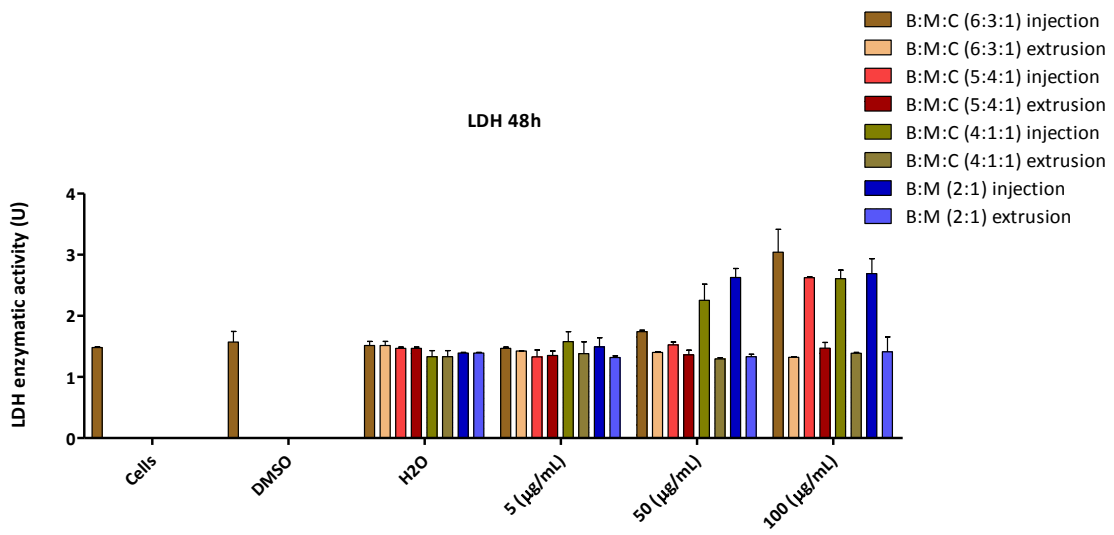


Figure 36. Evaluation of the cytotoxicity (cell membrane integrity) in L929 cell line induced by varying concentrations of DODAB:MO (2:1) and DODAB:MO:DC-Chol (6:3:1, 5:4:1, 4:1:1) prepared by ethanolic injection and film hydration followed by extrusion after 48h of incubation. Cells: cells alone; DMSO: cells incubated with 30 % DMSO; H2O: cells incubated with the volume of H2O used to incubate de liposomes to a final concentration of 100 µg/mL. The mean (+/−) SD was obtained from two independent experiments.

4.2 DNA/DODAB/C:MO:DC-Chol lipoplexes

Lipoplexes may affect differently cell membrane structure, metabolism or cell proliferation. The 293T cell line will be used to perform transfection assays so the citotoxicity induced by lipoplexes in L929 cell line was confirmed in 293T cell line. In fact, the results obtained for lipoplexes of charge ratio (+/-) 4.0 on cell viability show some differences when compared with the results obtained for liposomes. First of all, not all lipoplexes prepared with DODAB based liposomes promote higher cell viability has it was observe for DODAB liposomes earlier. In fact, lipoplexes prepared with DODAB:MO:DC-Chol with 6:3:1 and 4:1:1 ratios and produced by ethanolic injection, appear to be the most harmfull for cell metabolism. Lipoplexes prepared with DODAB:MO with 2:1 ratio and produced by ethanolic injection also have a great effect in cell viability but not to the same extent. Yet, lipoplexes prepared with DODAB:MO:DC-Chol liposomes produced by ethanolic injection don't have such a pronounced effect on cell survival (Figure 37).

As to the toxicity displayed by lipoplexes prepared with DODAC liposomes, the difference in cell viability between the two methods of liposome production don't seem to be so pronounced as in DODAB based liposomes. It is important to state that lipid concentration varies a little according to the amount of cationic lipids included in the liposomal formulation, in order to obtain lipoplexes of charge ratio (+/-) 4.0, since the amount of pDNA used in the lipoplex production is constant.

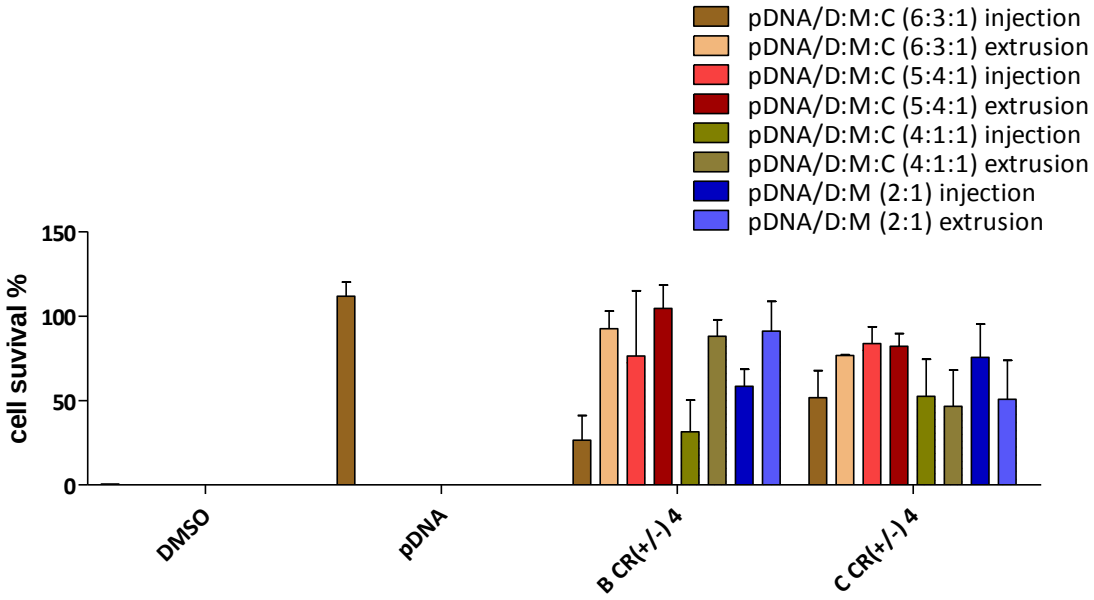


Figure 37. Evaluation of the cytotoxicity (metabolic assay) in 293T cell line induced by lipoplexes of pDNA/DODA(X):MO (2:1) and pDNA/DODA(X):MO:DC-Chol (6:3:1, 5:4:1, 4:1:1), based on liposomes produced by ethanolic injection and film hydration followed by extrusion, at charge ratio (+/-) 4.0 after 48h of incubation. pDNA: cells incubated with 1µg/µL of plasmid DNA; Cells: cells alone. B CR (+/-) 4.0: pDNA/DODAB:MO and pDNA/DODAB:MO:DC-Chol lipoplexes. C CR (+/-) 4.0: pDNA/DODAC:MO and pDNA/DODAC:MO:DC-Chol lipoplexes. The mean (+/−) SD was obtained from two independent experiments.

LDH enzyme activity is higher when cells contact with lipoplexes prepared with DODAB:MO:DC-Chol with 6:3:1 and 4:1:1 ratios and DODAB:MO 2:1 ratio and produced by ethanolic injection, indicating that these lipoplexes have a greater effect in cell membrane integrity and, therefore, greater effect in cell viability. These results are in accordance with the results obtained by MTT assay. Lipoplexes prepared with DODAC:MO:DC-Chol 6:3:1 and 5:4:1 and produced by both methods appear to cause

reduction of LDH enzyme activity and therefore, have less impact in cell viability (Figure 38).

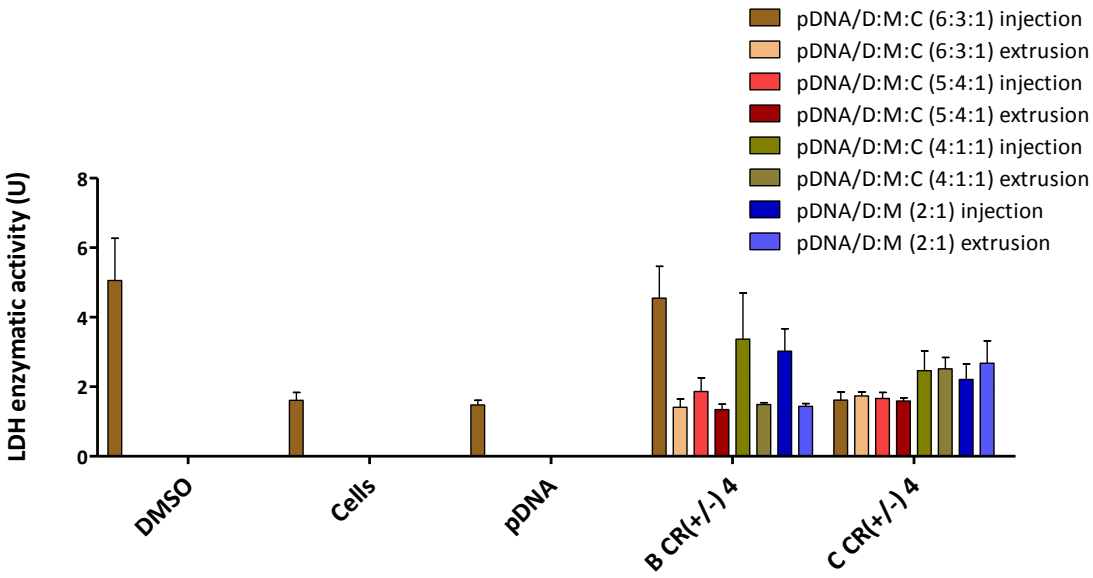


Figure 38. Evaluation of the cytotoxicity (cell membrane integrity) in 293T cell line induced by lipoplexes of pDNA/DODA(X):MO (2:1) and pDNA/DODA(X):MO:DC-Chol (6:3:1, 5:4:1, 4:1:1), based on liposomes produced by ethanolic injection and film hydration followed by extrusion, at charge ratio (+/-) 4.0 after 48h of incubation. DMSO: cells incubated with 30 % DMSO; Cells: cells alone. pDNA: cells incubated with 1µg/µL of plasmid DNA; Cells: cells alone. B CR (+/-) 4.0: pDNA/DODAB:MO and pDNA/DODAB:MO:DC-Chol lipoplexes. C CR (+/-) 4.0: pDNA/DODAC:MO and pDNA/DODAC:MO:DC-Chol lipoplexes. The mean (+/−) SD was obtained from two independent experiments.

It seems that for DODAC based lipossomes and lipoplexes, the DODAC:MO:DC-Chol (5:4:1) formulation is less toxic, especially the extruded liposomes and lipoplexes produced with these. In DODAC based liposomes, this formulation is the one with greater amount of MO ($X_{MO=}0.4$). Previous work with DODAB:MO system showed that higher content of MO reduced the citotoxicity levels in more than one cell line (Neves Silva *et al*., 2012), in both liposomes and lipoplexes, which agrees with the results of this work, even with the inclusion of DC-Chol ($X_{DC\text{-}Chol=}0.1$) in the (5:4:1) formulation. Furthermore, the reduction of MO content ($X_{MO}\approx0.16$) and inclusion of higher content of DC-Chol ($X_{DC\text{-}Chol}\approx0.16$) in the liposomal formulation (4:1:1) causes toxicity values comparable to the DODAC:MO (2:1) liposomes. Indeed, in previous reports, it was observed in some cell lines that DC-Chol induced reduced toxicity when compared with

commercial reagent Lipofectamine (Gao and Huang, 1991). The levels of toxicity seem to be a little higher only with liposomes and lipoplexes prepared with DODAC:MO:DC-Chol (4:1:1) produced by ethanolic injection. This might be explained, as mentioned before, by the preparation method itself, since the produced vesicles have different structures and variable size. Moreover, the presence of ethanol in the solution might be an issue since the evaporation of the organic solvent when injecting the lipids into the aqueous solution might not be complete.

DODAB based liposomes and lipoplexes seem to cause less toxicity than the DODAC based liposomes and lipoplexes. Also, the diference in toxicity induced by the two liposome preparation methods is greater than the one observed for DODAC based liposomes. The two counter ions, Bromide and Chloride, must influence differently parameters such as particle interaction with the cell membrane and pDNA compaction. It would be interesting to further explore this topic in future studies.

5. Transfection

Transfection efficiency was evaluated by the detection of β-galactosidase, the protein encoded in the pSV-β-gal plasmid. As reporter gene, β-galactosidase can be detected by a colorimetric assay. The transfection efficiency of pDNA/ DODAB/C:MO and pDNA/DODAB/C:MO:DC-Chol lipoplexes at charge ratio (+/-) 4.0, prepared with liposomes produced by ethanolic injection and film hydration method followed by extrused was evaluated and compared with that obtained with the commercial reagent EzWay. As illustrated in figure 39, DODAC based lipoplexes seem to present better results in terms of transfection efficiency than DODAB based lipoplexes. Furthermore, lipoplexes prepared with extruded DODAC liposomes also have enhanced tranfection efficiency when compared to the ones prepared with DODAC liposomes produced by ethanolic injection. However, in DODAB based lipoplexes, the effect seems to be quite the opposite. The lipoplexes prepared with ethanolic injection liposomes seem to yield better results. The results obtained with DODAC based lipoplexes are in the same order of magnitude as EzWay ™. The pDNA/DODAC:MO:DC-Chol (4:1:1) lipoplexes was the formulation that promoted higher transfection efficiency, even higher than the commercial system, followed by (6:3:1) and (5:4:1)

formulations. In fact, the inclusion of DC-Chol in the liposomal formulations appears to result in improved transfection efficiency, since in DODAC based liposomes, the formulations containing DC-Chol showed better results when compared with DODAB:MO (2:1). Although the DODAB based system was associated with lower transfection efficiency, contrary to the DODAC system, ethanolic injection liposomes resulted in better transfection efficiency. The inclusion of DC-Chol also appears to improve transfection when comparing DODAB:MO (2:1) with DODAB:MO:DC-Chol.

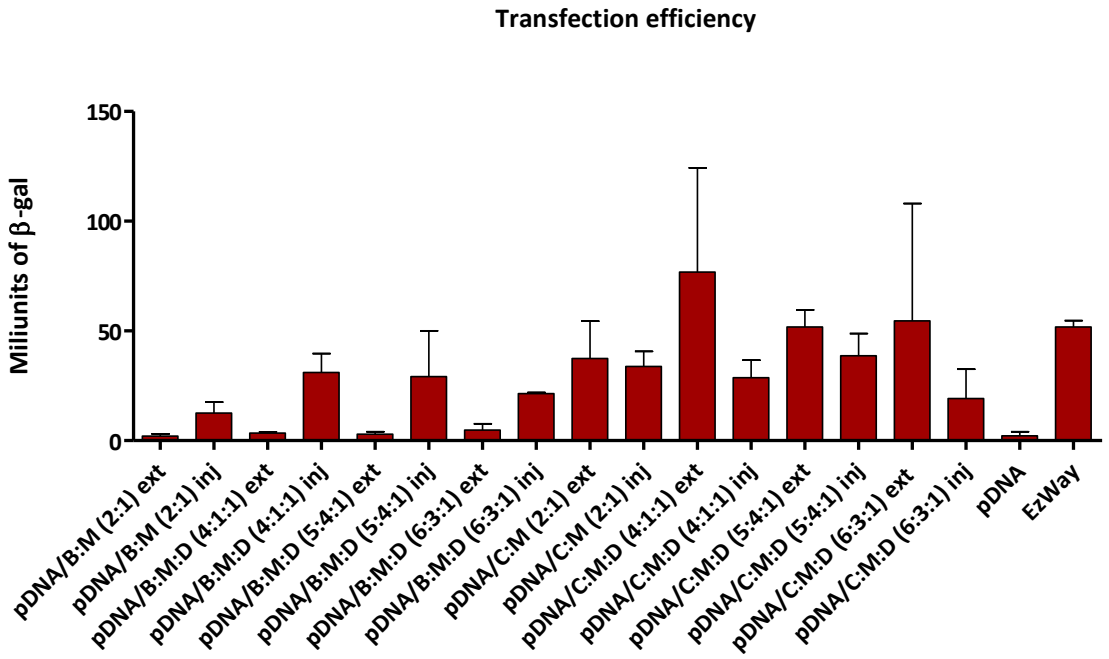


Figure 39. Transfection efficiency of 293T cells by lipoplexes of pDNA/DODA(X):MO (2:1) and pDNA/DODA(X):MO:DC-Chol (6:3:1, 5:4:1, 4:1:1), based on liposomes produced by ethanolic injection and film hydration followed by extrusion, at charge ratio (+/-) 4.0 with 1.0 µg pDNA/well after 48h of incubation. Transfected pDNA encoded the β-galactosidase gene whose activity was evaluated by a colorimetric assay after 48 h of incubation. pDNA: cells incubated with free pDNA; EzWay: cells transfected using EzWay ™ as lipofection agent. The mean (+/−) SD was obtained from three independent experiments.

In figure 40 is depicted the amount of B-galactosidas per amount of proteins in the transfected samples. The amount of proteins in the transfected samples was quantified by the Bradford method. This was done in order to normalize the transfection assay results for varying cell numbers which could result of citotoxicity, for example. The same tendency observed in the transfection results can be observed.

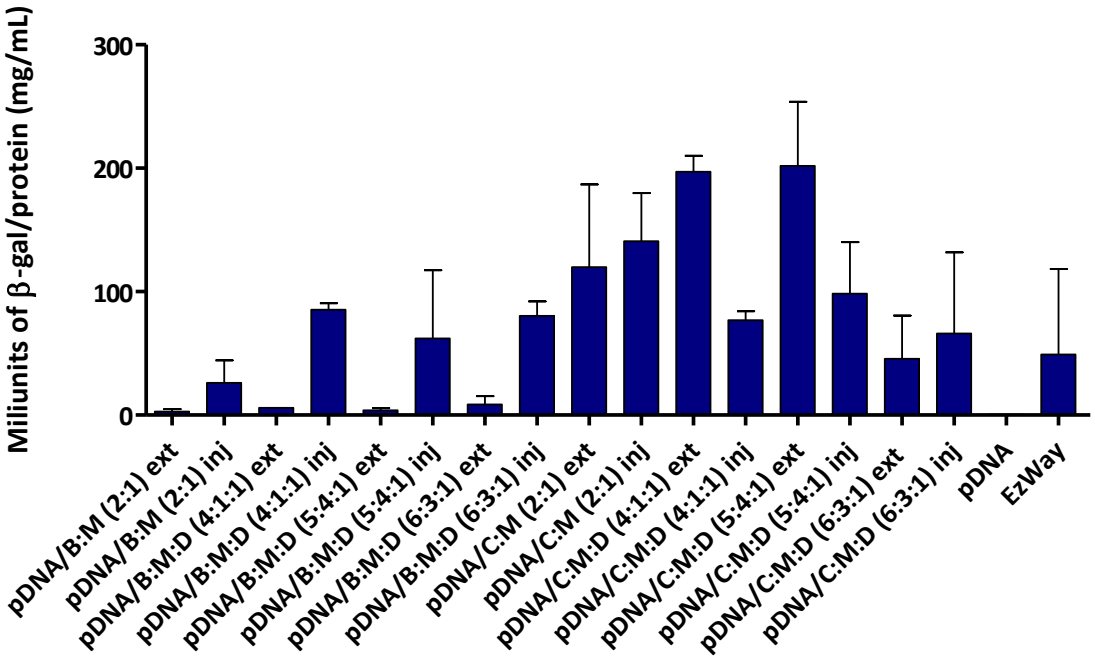


Figure 40. Quantification of B-gal miliunits in total protein content of 293T cells transfected with lipoplexes of pDNA/DODA(X):MO (2:1) and pDNA/DODA(X):MO:DC-Chol (6:3:1, 5:4:1, 4:1:1), based on liposomes produced by ethanolic injection and film hydration followed by extrusion, at charge ratio (+/-) 4.0 with 1.0 µg pDNA/well after 48h of incubation. Protein quantification of transfected cells was performed by the Bradford method. pDNA: cells incubated with free pDNA; EzWay: cells transfected using EzWay ™ as lipofection agent. The mean (+/−) SD was obtained from three independent experiments.

Comparing DODAB and DODAC based lipoplexes without the inclution of DC-Chol, the transfection efficiency is in the same order of magnitude for lipoplexes prepared with extruded liposomes. However, the inclusion of DC-Chol has different effects on DODAB and DODAC based lipoplexes. When including DC-Chol in the liposomal formulation of DODAC bases lipoplexes, they improved transfection efficiency, which is not observed for DODAB based lipoplexes. The method of liposome preparation shows different results for DODAB and DODAC based liposomes, with or without the inclusion of DC-Chol. These different results might be explained with different phase transition behaviours in DODAB and DODAC, but further studies like *Differential scanning calorimetry* are needed to assess this hypothesis.

Transfection experiments were also performed in L929 cells line in order to verify if the lipoplexes could transfect efficiently a different cell line. The experiment

was performed in the same conditions described for 293T cell line. The transfection efficiency of pDNA/DODA(X):MO and pDNA/DODA(X):MO:DC-Chol lipoplexes prepared with liposomes produced by ethanolic injection and film hydration method followed by extrused was evaluated and compared with that obtained with the commercial reagent EzWay ™ at charge ratio (+/-) 4.0. However, in these experiments, transfection efficiency was very poor, for both the commercial reagent and for the lipoplexes characterized in this study (data not shown). This shows that transfection efficiency not only depends on the liposomal formulation on which the lipoplexes are prepared but also on the cell type. This might be explained by the nature of the entry pathway of the lipoplexes into the cell according to the endocytic machinery (Rejman *et al*., 2005).
The exchange of the counterion bromide for chloride arises as a beneficial trade in terms of tranfection efficiency. The differences that emerge with counterion exchange are also reflected in the inclusion of DC-Chol in the liposomal formulation. This inclusion appears to be more beneficial in DODAC based liposomes. However, further studies are required in order to understand why the liposome preparation methods of the liposomes used to prepare the lipoplexes affect in such different ways the transfection efficiency. It would be interesting to study how this difference is reflected in lipoplex structure, internalization and lipoplex destabilization.

IV .Conclusion and Future perspectives

This work was focused on developing and characterizing DODAB/C:MO:DC-Chol lipoplexes as new and effective non-viral vectors suitable to perform efficient transfection.

In previous studies, DODAB:MO based lipoplexes showed promising results as non-viral vectors and the use of MO as helper lipid was proven successful (Neves Silva *et al*., 2012). Furthering these previous results by our research group, three major variables were studied in the present work: counter ion exchange, inclusion of DC-Chol in the liposomal formulation and liposome preparation methods.

It was thought that the exchange of the counterion bromide in DODAB for chloride in DODAC would possible influence liposome properties, which was indeed verified in this study. DODAB:MO (2:1) liposomes presented a larger mean size compared to DODAC (2:1) liposomes, irrespective of being produced either by ethanolic injection or by film hydration followed by extrusion. The inclusion of DC-Chol in DODAC based liposomes produced by film hydration followed by extrusion, however, did not have a significant effect on their mean size.

The ζ-potencial values of the liposomes seem to vary with the liposomes preparation method. DODAC based liposomes show higher ζ -potencial values for liposomes produced by ethanolic injection. For DODAB based liposomes, the same trend seems to occur, although the difference between the values displayed by the two methods is narrower.

The counter ion exchange also influenced the pDNA complexation with the liposomes, as studied by FRET and DLS measurements. DODAC:MO (2:1) seemed to complex with pDNA at a lower charge ratio and the complexation efficiency of DODAC:MO (2:1) was higher. Also, the lipoplexes mean size of DNA/DODAC:MO (2:1) lipoplexes was slightly smaller than the mean size of DNA/DODAB:MO (2:1) lipoplexes. The complexation of DNA/DODAC:MO:DC-chol lipoplexes was in agreement with the results obtained for DNA/DODAC:MO (2:1), although DNA/DODAC:MO:DC-Chol (5:4:1) was the most efficient formulation in incorportating the plasmid DNA. The mean size and ζ-potencial measurements of the lipoplexes seem to corroborate this observation.

Furthermore, in the presence of higher concentrations of NaCl, DODAC:MO (2:1) liposomes produced by extrusion and DNA/DODAC:MO lipoplexes prepared with these liposomes seemed to be more stable than the DODAB:MO (2:1) liposomes and DNA/DODAB:MO (2:1) lipoplexes. The inclusion of DC-Chol in DODAC based liposomes and lipoplexes had hardly any influence in the particles stability. This is valid for both mean size and Z-potential of the particles.

The exposure of DODAB/C:MO and DODAC:MO:DC-Chol liposomes to serum seems to be slightly more destabilizing to DODAC:MO (2:1) liposomes than to DODAC:MO (2:1) liposomes in terms of affecting their mean size. DC-Chol inclusion in DODAC based liposomes does not seem to cause significant effect when compared to DODAC:MO liposomes. The same trends seem to apply in lipoplex destabilization in the presence of serum.

Regarding citotoxicity, DODAC based liposomes and lipoplexes seem to induce more toxicity than the DODAB based liposomes and lipoplexes. The liposome preparation method also modulates toxicity. This difference is more pronounced in DODAB based liposomes. DC-Chol appears to exert the same effect in cell toxicity when included in either DODAB or DODAC based liposomes and lipoplexes.

Concerning transfection efficiency, the liposome preparation method used leads to different transfection results. DNA/DODAC:MO and DNA/DODAC:MO:DC-Chol lipoplexes produced by extruded liposomes yield better transfection results. However, in DNA/DODAB:MO and DNA/DODAC:MO:DC-chol lipoplexes, the opposite effect was registered. Again, the differences inherent to the preparation methods seem to be more noticeable in DODAB based lipoplexes. The addition of DC-Chol to the formulation seemed to have different effects in pDNA/DODAB:MO:DC-Chol and pDNA/DODAC:MO:DC-Chol lipoplexes. pDNA/DODAC:MO:DC-Chol lipoplexes seem to have enhanced tranfection efficiency when compared to pDNA/DODAC:MO lipoplexes. For DODAB based lipoplexes, only pDNA/DODAB:MO:DC-Chol 4:1:1 and 5:4:1 lipoplexes prepared with liposomes produced by ethanolic injection displayed better results than pDNA/DODAB:MO(2:1) liposomes.

The differences displayed by DODAB and DODAC based lipoplexes still require further research. The different efficiency in transfection may be due to physicochemical characteristics such as differences in the fluidity of the system.

Therefore, techniques like DSC should be employed in order to confirm this hypothesis. Also, further optimization studies on the inclusion of DC-Chol in the liposomal formulations are needed.

Interestingly, DODAC based lipoplexes, which yielded better transfection results, also seem to induce higher levels of citotoxicity. Moreover, DNA/DODAC:MO:DC-Chol 5:4:1 and 4:1:1 lipoplexes were the most efficient formulations in transfection. DNA/DODAC:MO:DC-Chol 4:1:1 lipoplexes displayed higher levels of toxicity and lower DNA complexation efficiency than the DNA/DODAC:MO:DC-Chol 5:4:1 lipoplexes. Also, the mean size of 4:1:1 lipoplexes seems to be slightly less stable in the presence of NaCl and serum. However, transfection efficiency levels are slightly higher in 4:1:1 lipoplexes. This might be due to differences on the lipoplexes interaction with the cell membrane.

The morphology and structure of lipoplexes is an important aspect to consider when it comes to transfection efficiency because of the influence exerted by the chemical nature of the constituents and also because of the surrounding environment. Moreover, there are other aspects of great significance that determine the effectiveness of vector internalization and, eventually, successful transgene expression. Taking this into consideration, the structure of the lipoplexes is a fundamental aspect to study in the future, as well as the way this structure might be shaped in accordance to different proportions of the constituents and how this might influence lipoplex-cell interaction. Furthermore, internalization studies are also in order to unveil how the lipoplexes enter the cell.

In the long term, DODAC:MO:DC-Chol lipoplexes appear as a new and promising non viral vector.

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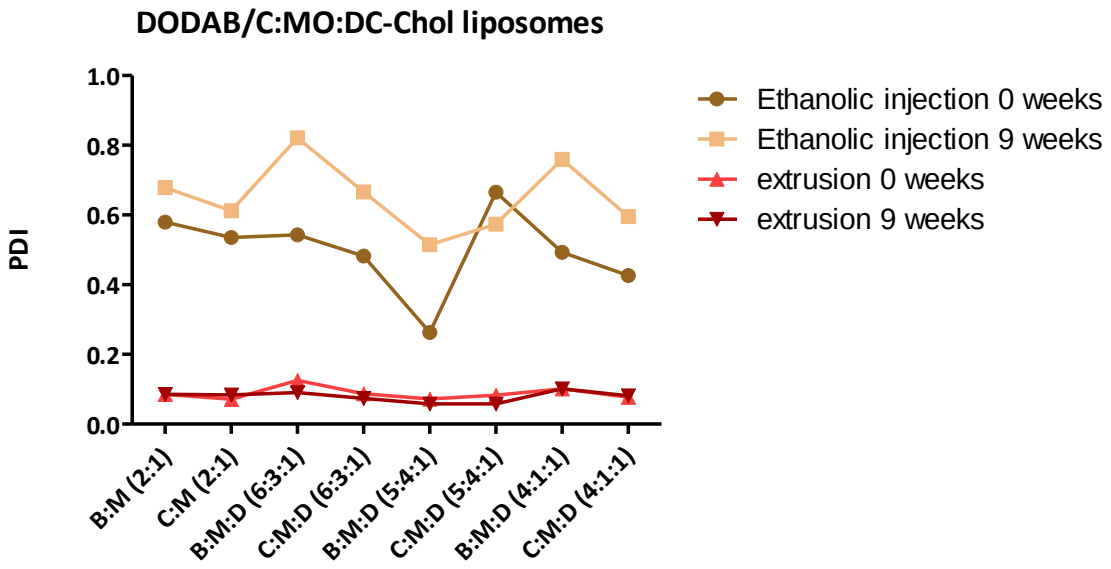
		Mean size (nm)	PDI	Pop. 1	%	Pop. 2	%	Pop. 3	%
Ethanolic injection	B:M:D (6:3:1)	214,5 ± 2,828	0,543 ± 0,115	312,9 ± 25,31	96,2 ± 1,1	4998 ± 511,9	3,8 ± 1,1	0	0
	B:M:D (5:4:1)	134,4 ± 1,115	0,263 ± 0,003	183,3 ± 3,523	100	0	0	0	0
	B:M:D (4:1:1)	122,5 ± 67,44	0,493 ± 0,168	108,6 ± 22,58	72,7 ± 15,8	1714 ± 2718	15,4 ± 13,8	5,172 ± 6,563	6,5 ± 1,2
	B:M (2:1)	269,6 ± 39,74	0,580 ± 0,02	246,4 ± 13,08	93,8 ± 8,8	2493 ± 3525	6,3 ± 8,8	0	0
	C:M:D (6:3:1)	95,57 ± 4,744	0,482 ± 0,035	157 ± 13,99	87,7 ± 10,9	1809 ± 3096	8,1 ± 5,9	2810 ± 2574	4,2 ± 5,6
	C:M:D (5:4:1)	97,76 ± 5,683	0,665 ± 0,176	214,8 ± 137,1	63,8 ± 28,7	1655 ± 2496	30,2 ± 23,5	10,31 ± 9,119	3,5 ± 3,2
	C:M:D(4:1:1)	108,9 ± 0,8485	0,426 ± 0,012	148,1 ± 1,061	90,8 ± 3,5	4450 ± 273,7	6,3 ± 0,6	12,03 ± 17,01	2,9 ± 4,1
	C:M (2:1)	73,66 ± 0,02828	0,535 ± 0,002	181,8 ± 8,980	67,8 ± 0,4	34,17 ± 2,553	29,7 ± 0,3	4881 ± 152	2,4 ± 0
Film hydration followed by extrusion	B:M:D (6:3:1)	135,4 ± 0,8505	0,125 ± 0,015	151,8 ± 1,380	100	0	0	0	0
	B:M:D (5:4:1)	131,2 ± 1,002	0,072 ± 0,015	142,9 ± 1,069	100	0	0	0	0
	B:M:D (4:1:1)	131,2 ± 0,6557	0,101 ± 0,013	147,3 ± 2,401	100	0	0	0	0
	B:M (2:1)	132,8 ± 0,9247	0,085 ± 0,013	145,3 ± 2,614	100	0	0	0	0
	C:M:D (6:3:1)	112,4 ± 1,419	0,087 ± 0,004	123,3 ± 2,219	100	0	0	0	0
	C:M:D (5:4:1)	125,7 ± 0,3	0,083 ± 0,002	137,8 ± 0,4509	100	0	0	0	0
	C:M:D(4:1:1)	106,8 ± 0,7095	0,077 ± 0,016	116,8 ± 1,229	100	0	0	0	0
	C:M (2:1)	109,5 ± 1,1	0,071 ± 0,002	119,2 ± 1,422	100	0	0	0	0

Table 1. Size and PDI values of DODAB/C:MO and DODAB/C:MO:DC-Chol liposomes produced by ethanolic injection and film hydration followed by extrusion.



Comparison of the polidispersity index (PDI) of DODAB/C:MO (2:1) and DODAB/C:MO:DC-CHOL (6:3:1, 5:4:1 and 4:1:1) liposomes produced by ethanolic injection and by film hydration followed by extrusion over 9 weeks. B:M (2:1) – DODAB:MO liposomes with 2:1 ratio; C:M (2:1) – DODAC:MO liposomes with 2:1 ratio; B:M:D (6:3:1) – DODAB:MO:DC-Chol liposomes with 6:3:1 ratio; C:M:D (6:3:1) – DODAC:MO:DC-Chol liposomes with 6:3:1 ratio; B:M:D (5:4:1) – DODAB:MO:DC-Chol liposomes with 5:4:1 ratio; C:M:D (5:4:1) – DODAC:MO:DC-Chol liposomes with 5:4:1 ratio; B:M:D (4:1:1) – DODAB:MO:DC-Chol liposomes with 4:1:1 ratio; C:M:D (4:1:1) – DODAC:MO:DC-Chol liposomes with 4:1:1 ratio.

Time point		% serum	Mean size	PDI	Pop. 1	%	Pop. 2	%	Pop. 3	%
0h	liposomes	0	135,4 ± 0,8083	0,077 ± 0,015	148,3 ± 3,56	100	0	0	0	0
		30	206,9 ± 5,147	0,578 ± 0,009	398,6 ± 14,3	93,1 ± 5,2	1675 ± 2816	6,2 ± 4,5	1651 ± 2860	0,6 ± 1,1
		80	130,2 ± 5,187	1	640,7 ± 142,4	75,8 ± 3,6	40,5 ± 3,822	14,2 ± 0,9	1513 ± 2607	7,2 ± 0.5
3h		30	140,5 ± 1,531	0,568 ± 0,008	257,9 ± 3,772	92,3 ± 1.1	28,18 ± 7,191	6,3 ± 1,3	2,465± 4,27	1,3 ±2,3
		80	51,99 ± 0,5415	1	372,4 ± 46,35	62,5 ± 0,9	35,63 ± 2,56	23,4 ± 2,4	7,458 ± 0,1197	12,8 ± 0,2
6h		30	140,4 ± 1,607	0,563 ± 0,005	244,6 ± 4,508	90,4 ± 0,4	20,55 ± 3,065	5,9 ± 0,9	7,031 ± 0,8731	3,7 ± 0,8
		80	62,24 ± 0,5948	1	495,9 ± 39,96	63,1 ± 0,9	38,35 ± 1,498	22,3 ± 0,7	7,684 ± 0,1731	12,4 ± 0,6
24h		30	138,5 ± 1,058	0,571 ± 0,005	256,1 ± 4,882	90,3 ± 2,7	20,14 ± 9,267	6,4 ± 0,3	19,93 ± 26.56	3,2 ± 2,8
		80	101,8 ± 1,457	1	654,3 ± 15,13	71,3 ± 0,7	43,31 ± 3,175	18,5 ± 1,7	7,595 ± 0,2474	9,2 ± 0,4
0h	lipoplexes C.R. (+/-) 4,0	0	175,7 ± 1,422	0,106 ± 0,014	199,2 ± 1,704	100	0	0	0	0
		30	149,9 ± 1,562	0,658 ± 0,008	309,4 ± 5,341	87,4 ± 3,3	42,54 ± 12,5	8,7 ± 0,3	6,161 ± 5,383	4 ± 3,5
		80	64,85 ± 1,106	1	394,7 ± 15,41	70,6 ± 0,6	37,31 ± 3,635	19,3 ± 1,4	7,68 ± 0,4862	10,1 ± 1,2
3h		30	166,8 ± 0,3055	0,608 ± 0,008	334,9 ± 8,929	90,6 ± 2,6	25,04 ± 6,664	7,8 ± 0,2	17,7 ± 30,65	0,8 ± 1,4
		80	96,91 ± 5,088	1	550,2 ± 14,19	72,4 ± 1,7	39,09 ± 5,652	16,2 ± 1,0	8,259 ± 0,9373	9,9 ± 1,2
6h		30	166,9 ± 3,061	0,608 ± 0,008	327,5 ± 14,64	90,4 ± 2,8	32,65 ±9,515	7,8 ± 0,6	2,931 ± 5,077	1,9 ± 3,2
		80	74,72 ± 0,1501	1	494,2 ± 17,85	72,1 ± 0,3	35,47 ± 2,895	18,5 ± 1.0	7,189 ± 0,5048	9,4 ± 1,1
24h		30	177,8 ± 1,721	0,62 ± 0,005	344,3 ± 2,417	92,5 ± 1,9	27,99 ± 6,645	6,4 ± 0,5	2,219 ± 3,843	1,1 ± 2,0
		80	101,9 ± 0,8	1	712,6 ± 88,57	71,9 ± 1,8	37,62 ± 4,144	16 ± 1,2	7,965 ± 0,5619	9,8 ± 1,0

Table 2. Size and PDI of DODAB:MO (2:1) liposomes and DNA/DODAB:MO (2:1) lipoplexes incubated with 30% and 80% serum at different time points for 24h.

Time point		% serum	Mean size	PDI	Pop. 1	%	Pop. 2	%	Pop. 3	%
0h	liposomes	0	124,2±0,7234	0,095±0,018	137,9±1,389	100	0	0	0	0
		30	80,44±0,7113	0,602±0,002	183,3±4,822	81,8±2	27,62±7,418	11,9±1,6	7,469±0,4436	6,3±1,2
		80	47,35±0,1457	1	336,4±5,437	63,2±5,3	39,01±9,238	24,4±3,4	7,746±0,5095	12,4±1,8
3h		30	125,3±1,804	0,555±0,015	238,8±26,32	87,4±3,3	26,91±16,85	9,1±1.6	1684±2903	3,5±2,2
		80	54,54±2,874	1	636±109	60,5±3,4	36,51±5,385	25,7±2,4	7,525±0,5942	12,5±1,9
6h		30	165,2±0,9849	0,555±0,004	303,3±18,88	94±5,3	15,93±17,94	3,8±3,3	1523±2632	2,2±2
		80	123,8±16,05	1	1717±660,6	65,2±2	33,13±1,783	18,4±2,7	65,82±101,2	10,5±0,8
24h		30	214,8±3,387	0,567±0,043	340,3±96,64	77,3±15,5	1778±2249	19,2±18,8	1650±2828	2,4±2,5
		80	543,2±158,60	1	4557±573,7	58±17,5	31,05±3,004	17,3±6	52,17±77,71	11,1±3,3
0h	lipoplexes C.R. (+/-) 4,0	0	165,1±3,512	0,234±0,002	214,9±12,29	98,1±1,7	2505±2256	1,9±1,7	0	0
		30	104,2±0,3215	0,559±0,006	202,7±2,179	85,7±0,7	25,08±13,95	8,3±1,4	19,95±21,32	6±0,9
		80	53±0,6122	1	277,5±11,45	69,4±1,5	33,96±1,571	20,2±1,3	7,267±0,3049	10,4±0,2
3h		30	113,9±1,308	0,6±0,005	219,2±4,65	90,4±1,3	19,14±6,402	8,3±0,6	3,405±3,409	1,3±1,9
		80	116±9,872	1	860,2±78,43	60,3±4	44,03±3,124	21,2±1	1368±2356	10,7±0,5
6h		30	202,2±2,663	0,553±0,018	397,7±25,06	95,6±3,8	30,99±26,92	4,4±3,8	0	0
		80	288,9±54,36	1	4275±564,1	50,7±13	34,93±4,667	20,2±3,8	636±591,6	15,2±6,5
24h		30	1727±124	0,746±0,006	3779±484	80±11,4	704,6±366	17,8±8,5	61,57±106,6	2,2±3,8
		80	391,6±74,91	1	4227±514,3	67,3±7,6	44,13±28,41	11,8±2,2	29,03±25,7	9,8±0,8

Table 3. Size and PDI of DODAC:MO (2:1) liposomes and DNA/DODAC:MO (2:1) lipoplexes incubated with 30% and 80% serum at different time points at different time points for 24h.

Time point		% serum	Mean size	PDI	Pop. 1	%	Pop. 2	%	Pop. 3	%
0h	liposomes	0	117,7±0,2	0,075±0,011	128,1±1,06	100	0	0	0	0
		30	59,9±1,427	0,619±0,052	143,7±4,007	83,5±4,6	14,54±5,877	14,1±3,3	2,429±4,207	2,4±4,2
		80	45,93±5,649	0,806±0,168	208,9±0,2	64,4±0,7	31,18±2,462	22,5±0,7	7,502±0,3132	13,1±0,6
3h		30	200,2±1,2	0,587±0,002	389,7±16,49	91,9±3,3	1475±2507	5,4±1,7	2957±2564	2,7±2,3
		80	79,64±1,776	1	1275±112,1	60,5±6,9	40,82±6,545	22,7±2,5	95,16±151,8	12,9±1,8
6h		30	165,5±0,9539	0,527±0,005	297,6±12,62	99,5±0,9	1478±2560	0,5±0,9	0	0
		80	194,8±14,74	1	2763±441,5	71,6±1,8	37,69±3,941	15,4±1,4	7,666±0,284	9,4±0,1
24h		30	201,8±0,9018	0,537±0,005	404,2±47,43	96,3±5,2	1532±2609	3,2±4,3	1528±2647	0,5±0,9
		80	381,7±18,8	1	4060±299	66,7±11,1	33,41±3,721	14,3±3,4	7,374±0,1865	9,8±2,2
0h	lipoplexes C.R. (+/-) 4,0	0	139,7±1,058	0,098±0,021	156,9±3,493	100	0	0	0	0
		30	109,1±1,193	0,582±0,011	211,3±7,165	87,3±1,1	28,68±6,496	6,9±0,8	8,721±0,5406	5,8±0,7
		80	58,15±0,8356	1	286±6,793	72,4±2	34,49±4,452	17,6±1,4	7,595±0,0497	9,9±0,9
3h		30	174,1±2,974	0,598±0,006	329,5±11,11	90,3±1,7	32,74±8,228	6,2±0,2	1558±2684	3,5±1,9
		80	88,58±0,5963	1	1255±83,97	63,7±4,8	33,61±3,222	19,2±0,7	83,35±132	10,6±0,7
6h		30	420,4±13,88	0,654±0,029	952,9±131,3	86,4±1,8	102±2,668	9,4±1	16,16±8,044	2,6±0,4
		80	216,1±44,17	1	3013±450,7	68,3±5,2	43,79±10	15,4±3	7,837±0,5718	10,7±1,6
24h		30	1842±32,58	0,518±0,15	2907±381,5	97,7±3,9	128,6±222,7	2,3±3,9	0	0
		80	1049±258,2	1	3437±2963	42,9±15,2	23,96±15,24	23,7±3,2	224,1±375,5	18±3,5

Table 4. Size and PDI of DODAC:MO:DC-Chol (6:3:1) liposomes and DNA/DODAC:MO;DC-Chol (6:3:1) lipoplexes incubated with 30% and 80% serum at different time points for 24h

Time point		% serum	Mean size	PDI	Pop. 1	%	Pop. 2	%	Pop. 3	%
0h	liposomes	0	122±0,3	0,059±0,025	130,9±3,037	100	0	0	0	0
		30	66,76±2,127	0,613±0,014	153±2,326	84,3±4,7	17,36±7,83	11,1±0,9	4,715±4,096	4,6±4,1
		80	46,86±0,4262	1	273,3±34,2	63,9±2,9	35,18±7,137	23,3±1,9	7,536±0,4288	12,1±1,7
3h		30	102,7±0,6658	0,563±0,008	203,3±4,854	92,1±2,5	8,705±1,241	6,1±0,6	8,78±15,21	1,2±3,1
		80	99,83±4,382	1	1219±162,7	59,5±4,3	32,96±3,922	17,4±1,2	141,8±76,86	13,6±2,7
6h		30	133,2±1,286	0,526±0,004	242,3±4,592	91,9±2,4	14,68±8,492	5,1±0,4	14,37±18,99	3±2,7
		80	166,8±1,229	1	2815±276,2	62,6±2,2	35,9±3,625	18,3±1,5	91,3±144,8	10,7±0,7
24h		30	123,1±0,7403	0,436±0,001	187,8± 4,571	93,9±0,9	12,86±6,032	4,5±1,1	1507±2605	1,6±1,6
		80	328±32,39	1	4466±189,2	64,6±3,5	34,24±1,41	16,9±0,9	51,09±76,22	9,6±1
0h	lipoplexes C.R. (+/-) 4,0	0	145,2±1,007	0,106±0,008	164,2±2,352	100	0	0	0	0
		30	114,8±0,5292	0,581±0,004	210,2±1,443	90,3±1,4	19,75±2,117	8±0,4	3,676±3,238	1,7±1,6
		80	99,71±1,217	0,699±0,006	312,5±1,323	75,6±0,3	41,06±4,838	16,2±0,9	7,867±0,7119	8,2±0,7
3h		30	136±1,808	0,586±0,007	257,8±6,514	88,8±2,2	26,36±6,918	8,7±0,9	4,309±3,925	2,5±2,6
		80	108,7±1,801	1	708,6±170,1	71±3,7	46,37±2,924	18,3±1,4	7,787±0,349	7,9±0,4
6h		30	265,2±4,59	0,624±0,018	598,3±40,26	88,6±3,4	71,14±46,77	8±5,4	5,886±5,116	2,7±2,3
		80	100,2±0,2082	1	1186±122,6	59,1±1,9	103,4±62,53	17,9±1,8	89,56±90,7	14,1±1,6
24h		30	2110±100,1	0,601±0,05	3287±144,8	100	0	0	0	0
		80	774,7±131,4	0,91±0,091	232,4±176,4	37,7±7,7	135,2±169,9	34,2±4,7	41,81±58,45	22,2±2,1

Table 5. Size and PDI of DODAC:MO:DC-Chol (5:4:1) liposomes and DNA/DODAC:MO:DC-Chol (5:4:1) lipoplexes incubated with 30% and 80% serum at different time points for 24h.

Time point		% serum	Mean size	PDI	Pop. 1	%	Pop. 2	%	Pop. 3	%
0h	liposomes	0	117,6±0,3606	0,065±0,007	127,3±0,3055	100	0	0	0	0
		30	52,45±0,564	0,619±0,008	136,1±7,835	77±3,1	22,88±6,863	15,6±2,7	6,684±1,502	7,4±3
		80	44,89±0,1531	1	38,73±38,73	2,9±2,9	4,912±4,912	4,1±4,1	0,1216±0,1216	0,8±0,8
3h		30	142,4±2,751	0,512±0,026	250,8±11,34	94,2±1,2	26,7±15,17	5,8±1,2	0	0
		80	94,16±2,129	1	1258±332	61±1,1	37,34±2,854	21,2±2,2	7,561±0,03213	11,4±0,8
6h		30	153,3±0,9644	0,474±0,007	262,7±8,524	97±3,8	1478±2539	2±2,1	2,311±4,003	1±1,7
		80	256,1±37,36	1	3174±397,5	70,2±3	32,4±3,205	15,2±1,8	7,173±0,2526	8,5±0,3
24h		30	204,3±7,758	0,483±0,014	437,4±214,7	93,8±6,8	1594±2720	4±3,5	1517±2628	2,2±3,9
		80	450,2±91,41	1	3214±1133	60,9±24	1660±2812	17,8±11,9	13,54±9,961	11±3,7
0h	lipoplexes C.R. (+/-) 4,0	0	195,6±0,6028	0,261±0,011	260,9±30,35	98,3±2,9	1410±2442	1,7±2,9	0	0
		30	124,1±1,058	0,532±0,006	227,7±4,259	88,8±0,5	24,39±3,33	6,4±0,3	8,003±1,013	4,7±0,6
		80	58,52±0,2629	1	292,4±8,523	72,9±0,8	33,02±1,698	17,3±0,6	7,393±0,09993	9,8±0,3
3h		30	217,6±7,136	0,564±0,044	418,9±70,19	89,9±8,7	24,08±21,07	4,5±3,9	3069±2664	2,9±2,5
		80	73,81±3,833	1	762,2±83,78	64,7±1,4	37,23±7,47	21,1±2,5	43,04±61,19	10,5±1,4
6h		30	152,5±15,47	0,722±0,017	356,6±450,9	84,9±6,6	31,38±2159	9,3±10,4	7,877±2924	5,9±4,6
		80	59,31±33,06	1	509,1±381,3	63,8±2,1	37,45±5,556	23,9±4,1	7,369±34,05	12,3±1
24h		30	1790±151	0,789±0,081	3496±1197	75,9±14,6	1640±3136	15,1±8,8	11,93±4,888	4,8±3,4
		80	650,7±24,72	1	3319±503,8	43,4±11,1	58,36±5,801	23,1±4,6	278±1,02	16,7±2,3

Table 6. Size and PDI of DODAC:MO:DC-Chol (4:1:1) liposomes and DNA/DODAC:MO:DC-Chol (4:1:1) lipoplexes incubated with 30% and 80% serum at different time points for 24h.